CANCER CACHEXIA: EVALUATION OF BODY COMPOSITION AND IDENTIFICATION OF GENETIC MARKERS

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DECLARATION OF ORIGINALITY

I declare that the work described in this thesis was undertaken by me and the thesis was composed and referenced by me personally. This work has not been submitted for any other professional degree or professional qualification.

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3. **Tan BH**, Birdsell LA, Martin L, et al. Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer. Clin Cancer Res. 2009;15:6973-9. PMID: 19887488

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5. **Tan BH**, Fearon KC. Cytokine gene polymorphisms and susceptibility to cachexia. Curr Opin Support Palliat Care. 2010;4:243-8 PMID: 207334968

6. **Tan BH**, Ross JA, Kaasa S, et al. Identification of possible genetic polymorphisms involved in cancer cachexia: a systematic review. J Genet. 2011;90: 165-177. PMID: 21677406

7. **Tan BH**, Fladvad T, Braun TP, et al. P-selectin genotype is associated with the development of cancer cachexia. EMBO Mol Med. 2012 [Epub ahead of print]. PMID: 22473907

ABBREVIATIONS

ACE	Angiotensin converting enzyme
APPR	Acute phase protein reactant
APR	Acute phase response
AR	Androgen receptor
BMI	Body mass index
CAM	Cell adhesion molecule
CHF	Chronic heart failure
CI	Confidence interval
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CNTF	Ciliary neurotrophic factor
CRP	C-reactive protein
CT	Computed tomography
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FABP	Fatty acid binding protein
FDR	False discovery rate
FFA	Free fatty acid
FFM	Fat free mass
FM	Fat mass
GFAT	Glutamine:fructose-6-phosphate aminotransferase
GH	Growth hormone
GWAS	Genome wide association study
HBP	Hexosamine biosynthetic pathway
HR	Hazard ratio
HU	Hounsfield unit
HWE	Hardy-Weinberg equilibrium
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IQ	Interquartile

LD	Linkage disequilibrium
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinase
MT	Metallothionein
NF	Nuclear factor
NSCLC	Non-small cell lung cancer
OR	Odds ratio
PCR	Polymerase chain reaction
PG-SGA	Patient generated-subjective global assessment
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
QoL	Quality of life
REE	Resting energy expenditure
ROC	Receiver-operator characteristic
RNA	Ribonucleic acid
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPI	Triose phosphate isomerase
UCP	Uncoupling protein
UPP	Ubiquitin proteasome pathway
UTR	Untranslated region
VDR	Vitamin D receptor

ABSTRACT

Cachexia is characterised by a chronic wasting syndrome, involving loss of skeletal muscle tissue with or without adipose tissue, which is resistant to conventional nutritional support. The typical patient with advanced cachexia demonstrates loss of appetite, early satiety, severe weight loss, weakness, anaemia and fluid retention. Affected individuals are also likely to report/experience decreased quality of life, decreased levels of physical performance, increased levels of fatigue, increased risks of treatment failure (be it chemotherapy, radiotherapy or surgery), increased risks of treatment side effects, and an increased mortality rate. Cachexia is therefore an extremely important, yet often underappreciated, cause of cancer patient morbidity and mortality which requires urgent attention. However, due to the epidemic of obesity in Western Society, a substantial proportion of oncology patients at the start of palliative therapy now have a BMI in the overweight range and this can confound conventional measures used for risk stratification.

Cachexia in its advanced phase (where patients may have lost 20-30% of their body weight) is easily identified. However, at this stage, the primary initiating events are frequently compounded by secondary factors (e.g. prolonged patient bed rest), and it is often impossible to attempt any realistic form of intervention, either practical or (given the patient's almost imminent demise) ethically advisable. Thus, any systematic approach to the treatment of cachexia requires early identification of patients at risk of cachexia and the institution of prophylactic measures to attenuate its progression.

The main aims of this thesis are: 1) To assess if measures of body composition, specifically skeletal muscle, have any prognostic value, and to chart time course changes in regional

body fat and lean tissue compartments using pancreatic cancer as a model for cancer cachexia. 2) To identify genetic variants that may be associated with susceptibility of developing cancer cachexia by means of a candidate gene association study.

Using a novel method of assessing body composition using computed tomography (CT) scans, 56% of pancreatic patients (n = 111) were found to be sarcopenic at the time of diagnosis. A much greater rate of fat loss was observed as compared with muscle loss in the course of the cancer journey. The combination of sarcopenia and overweight/obesity was found to be a poor prognostic indicator (HR 2.07, 95%CI 1.23 – 3.50, p=0.006). Despite a substantial proportion of cancer patients at diagnosis now having a BMI in the overweight range, the previously noted tendency to muscle wasting continues with the majority of patients' sarcopenic at diagnosis. The combination of sarcopenia and overweight/obesity has been highlighted as a poor prognostic factor in cancer patients and should be considered in the stratification of cancer patients' entering clinical trials, systemic therapy or support care programs.

The candidate gene association study was carried out based on data from two preliminary studies identifying genes involved in the pathogenesis of cancer cachexia. A systematic literature review identified 184 polymorphisms in 92 genes that have potential as susceptibility biomarkers for cancer cachexia. A gene expression array (Affymetrix) examining muscle of cachetic versus non-cachetic patients revealed an 83 gene signature correlating with weight loss.

A total of 191 SNPs in 99 genes were selected for the candidate gene association analysis. The study was carried out in a cohort of 775 patients and significant results were validated in a separate cohort of 101 patients.

21 SNPs had significant associations with cachexia. However, only the C allele (minor allele frequency 10.7%) of the rs6136 (SELP) SNP was found to be associated with cachexia (weight loss >10%) both in the main study (OR 0.52, 95%C.I 0.29 – 0.93, p=0.026) and the validation study (OR 0.09, 95%C.I 0.01 – 0.98, p=0.035). Gene-group analysis was performed based on functional similarity according to gene ontology in the main study patients. Gene groups regulating appetite, glucocorticoid signalling, and mitogen activated protein kinases (MAPK) activity were associated with cachexia (weight loss >15% and CRP >10mg/l) (p=0.0008, p=0.018, and p=0.026 respectively).

Patients who possess the C-allele of the P-selectin (SELP) rs6136 polymorphism appear to be at reduced risk of developing cancer cachexia. The rs6136 polymorphism may prove to be a useful susceptibility biomarker. Grouping of candidate genes according to gene ontology revealed three groupings that were associated with the development of cachexia. Genes regulating appetite, glucocorticoid signalling and MAPK activity appear to be important in the pathogenesis of cachexia and should be further investigated.

CHAPTER 1

INTRODUCTION

1.1 Background

. . .the shoulders, clavicles, chest and thighs melt away. This illness is fatal. . .

(Hippocrates 460–370 BC)

The origin of the term 'cachexia' is from the Greek words *kakós* (bad) and *hexis* (condition or appearance) and throughout medical history has been associated with the gravely ill patient.

Cachexia typically manifests in chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), and chronic kidney disease (CKD). The cachectic state is particularly problematic, typified by poor prognosis and often associated with a lower response to therapy. Cachexia also impairs quality of life (QoL) and is a significant cause of morbidity and mortality (Morley *et al*, 2006).

1.2 Definition of cachexia

While cachexia has long been recognized as a syndrome associated with many illnesses, there is currently no universally agreed definition. Traditionally, the definition of cachexia is limited to an assessment of involuntary weight loss. Although this has face-validity as an approach to screening, it is not clear that this minimal approach is adequate to guide clinical care decisions (Giordano & Jatoi, 2005). It is also not clear to what extent weight loss alone is associated with adverse functional status. Poor physical function may relate to many factors, including reduced substrate supply (food intake), and loss of lean body mass; all of

which have been considered in the definition of cachexia (Fearon *et al*, 2006; Roubenoff *et al*, 1997).

Weight loss

Weight loss has been shown to be an independent variable that predicts mortality in patients with chronic diseases and weight loss cut-offs of between 4% and 10 %, and BMI thresholds of between 18.5 and 23.0kg/m^2 have been used in cancer, COPD and CHF to define cachexia (Anker *et al*, 2003; Fearon & Moses, 2002; Schols, 2002).

Nutritional factors

A reduced nutritional intake secondary to anorexia is frequently a feature of cachexia. Anorexia is common in cancer patients and a study of 66 cancer patients nearing the end of life showed that 61% had anorexia despite the fact that they were not receiving chemotherapy (Tranmer *et al*, 2003). Reduced calorie intake and anorexia has also been correlated with weight loss in patients with chronic disease (Wallace & Schwartz, 2002). It has been suggested that a reduced nutritional intake and/or anorexia should be a component in the definition of cachexia (Evans *et al*, 2008; Fearon *et al*, 2006).

Skeletal muscle and strength

Muscle wasting is important in the pathophysiology of cachexia and a major cause of fatigue in patients (Evans & Lambert, 2007). Grip strength was found to be lower in cancer patients with a weight loss of >10% versus weight loss of 5% – 10% (Fearon *et al*, 2006). In 1997, Roubenoff *et al.* suggested that cachexia be defined on the basis of lean body mass alone.

This did not gain wide acceptance in the scientific community as patients with age-related muscle loss could be labeled as cachetic without losing weight.

Systemic inflammation

Many of the factors in cachexia such as weight loss, muscle wasting, and anorexia have been related, at least in part, to the effects of systemic inflammation (Laviano *et al*, 2003; Stone, 2002). C-reactive protein (CRP), a widely used marker of pro-inflammatory activity, has been shown to distinguish groups with weight loss versus weight stable patients with cancer (Martin *et al*, 1999; Scott *et al*, 2002), although in a recent study there was no significant difference in CRP levels between cachetic and non-cachectic male cancer patients (Stephens *et al*, 2012). CRP has also been shown to be an independent variable of adverse prognosis in patients with cancer (Crumley *et al*, 2006a; Deans & Wigmore, 2005). Markers of systemic inflammation such as CRP have been included in a proposed composite definition of cachexia (Fearon *et al*, 2006).

A study of patients with advanced pancreatic cancer, showed that weight loss alone does not identify the full effect of cachexia and is not a prognostic variable (Fearon *et al*, 2006). However, by utilising a multi-profile definition of cachexia, they were able to define a population that had both a lower subjective and objective functional ability. The study proposed that cachexia is defined by a combination of 3 factors (i.e. weight loss >10%, CRP >10 mg/l, and food intake <1500 kcal per day).

Recently, in December 2006, a committee of scientists and clinicians met in Washington, DC, to reach a consensus on the definition of the constellation of abnormalities that have been grouped under the name cachexia and approved the following definition: "Cachexia is

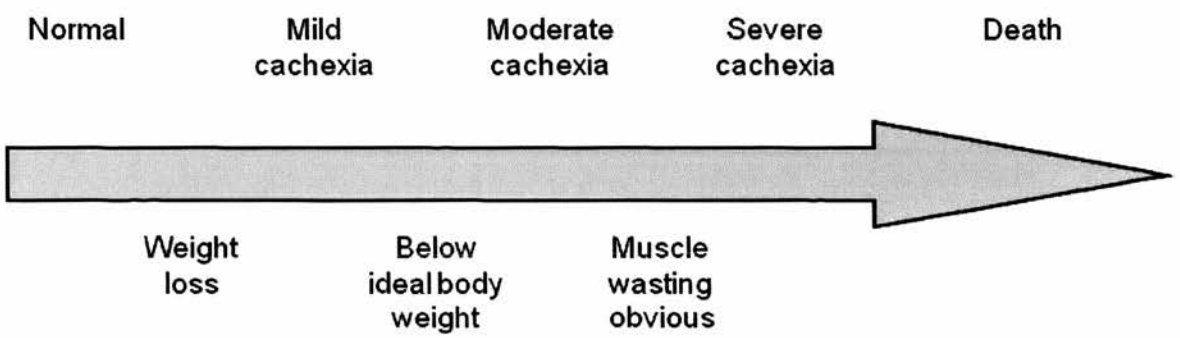
a complex metabolic syndrome associated with underlying illness and characterised by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults and growth failure in children. Anorexia, inflammation, insulin resistance and increased muscle breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and primary hyperthyroidism and is associated with increased morbidity" (Evans *et al*, 2008).

1.2.1 Classifying cancer cachexia

Cancer cachexia falls into a spectrum. Patients may first notice simple weight loss and then progress through degrees of severity to the point where they are depleted of energy reserves (fat), have gross muscle wasting, are immunocompromised, and will die primarily as a result of these issues (Figure 1.1).

Figure 1.1 The cachexia journey

Not all patients progress along the entire pathway



The recent consensus article on the definition of cachexia has suggested classifying the degree of cachexia as mild, moderate or severe, depending on whether the observed weight loss is >5%, >10% or >15%, respectively (Evans *et al*, 2008).

1.3 The changing demographics of cancer patients

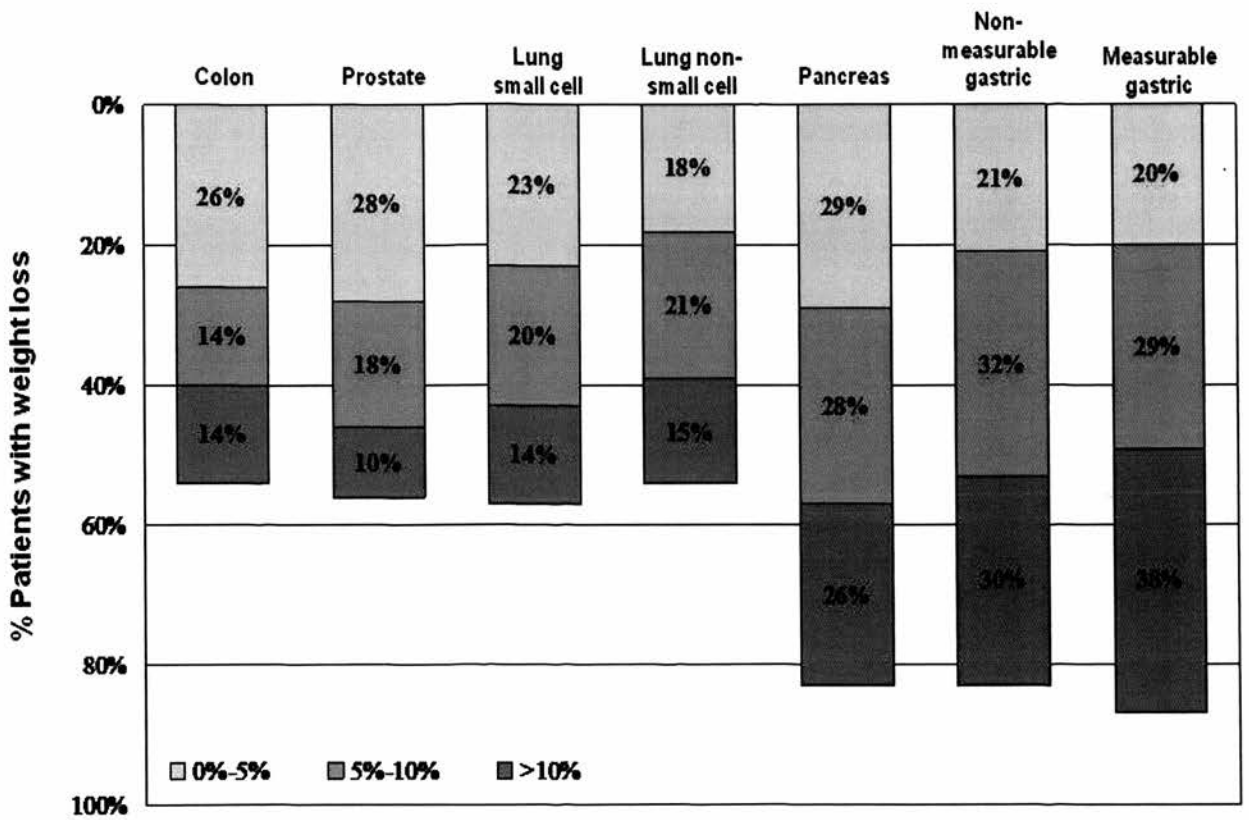
There is no doubt that cancer patients are becoming increasingly obese. For example, despite a predicted prevalence of weight loss affecting ≈50% (Teunissen *et al*, 2007), patients newly diagnosed to have incurable cancer commonly have a mean BMI greater than 25 (Irigaray *et al*, 2007). In obesity, however, a small degree of weight loss can mask a proportionately higher loss of skeletal muscle mass (Roubenoff, 2004). Indeed, a recent article has demonstrated that in advanced cancer patients with a mean BMI of 25.5, the prevalence of sarcopenia approaches 80% (Prado *et al*, 2008).

1.4 Cachexia in cancer

Approximately one quarter of all deaths in Western Society are due to cancer. Half of all patients with cancer lose some body weight; one third lose more than 5% of their original body weight and up to 20% of all cancer deaths are caused directly by cachexia (through immobility, cardiac/respiratory failure) (Skipworth *et al*, 2007). The incidence of weight loss upon diagnosis varies greatly according to the tumour site (Figure 1.2). The greatest incidence of weight loss is seen among patients with solid tumours, for example, gastric,

pancreatic, lung, colorectal, and head and neck (Dewys *et al*, 1980). 80% of the patients with pancreatic cancer have at least 10% weight loss at diagnosis and the cachexia syndrome is present in 25% (Fearon *et al*, 2006). The overall prevalence of weight loss in cancer patients may rise as high as 86% in the last 1–2 weeks of life (Teunissen *et al*, 2007).

Figure 1.2 Frequency and severity of weight loss in patients with advanced cancer attending for outpatient chemotherapy. (Derived from DeWys et al. 1980)



1.4.1 Impact of cancer cachexia

Survival

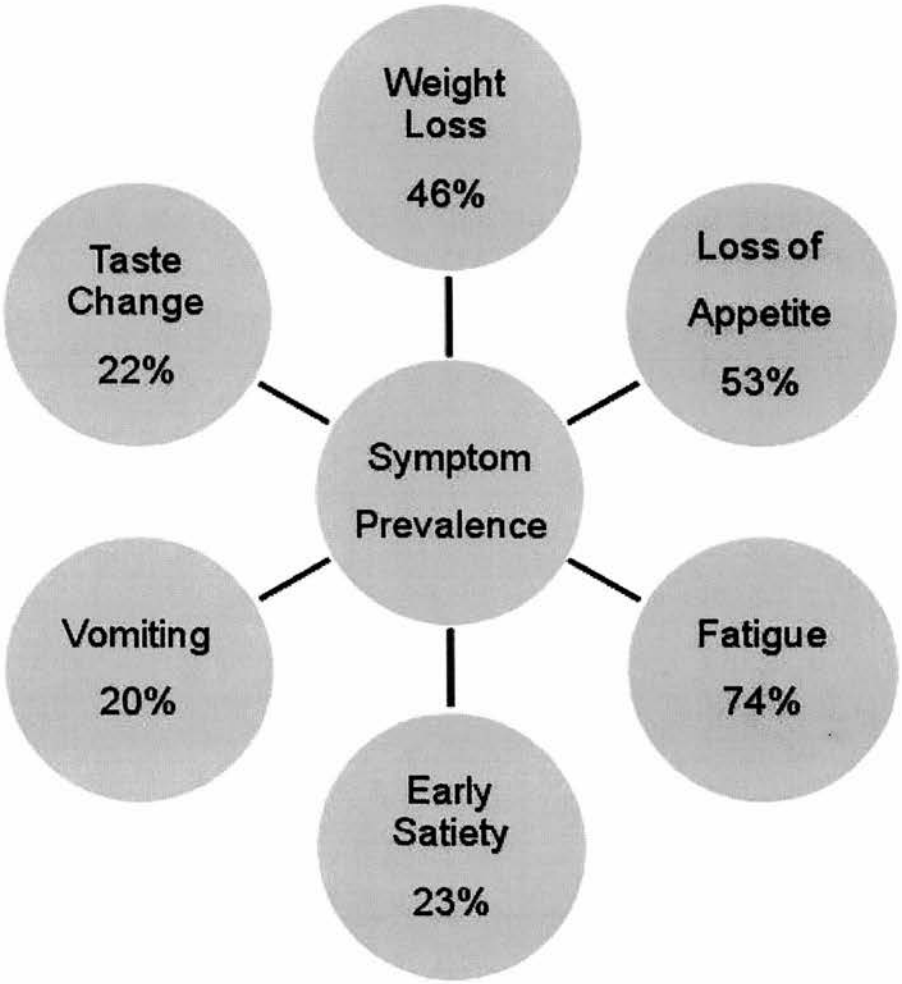
Based on the three-factor profile (weight loss, reduced food intake, and systemic inflammation) of cancer cachexia syndrome, pancreatic cancer patients who met at least two of the components had a significantly worse prognosis (Fearon *et al*, 2006). Alternatively, in a study of patients with oesophageal cancer, weight loss of more than 2.75% per month was found to be an independent prognostic indicator of decreased survival (Deans *et al*, 2007b).

Quality of life

Cachexia has a detrimental effect on a patient's QoL. Patients with cancer cachexia report altered body image, which impacts their emotions, spirituality, relationships, and social functioning. Lives are restricted and isolated, which is compounded by emotional distancing by carers and healthcare professionals (Hinsley & Hughes, 2007). These patients also experience anorexia and increased fatigue (Fearon *et al*, 2006; Teunissen *et al*, 2007) (Figure 1.3). Overall, this results in decreased performance status and QoL indices (Fouladiun *et al*, 2007).

The devastating effect of cachexia is further characterized by considering the free-living physical activity of patients. In a study examining patients using an electrical activity monitor worn over a period of 1 week, patients with cancer cachexia demonstrate a 40% reduction in the level of physical activity (Dahele *et al*, 2007).

Figure 1.3 Symptom prevalence in patients with incurable cancer. (Derived from Teunissen et al. 2008)



Treatment

Cachexia has a significant impact on cancer treatment. Dewys *et al.* (1980) defined weight loss of more than 5% prior to the onset of chemotherapy as the defining point for risk of poor response to therapy and shortened survival. A separate study in patients with lung cancer showed that patients with weight loss more frequently failed to complete at least three cycles of chemotherapy and had decreased survival duration (Ross *et al*, 2004).

Costs to Society

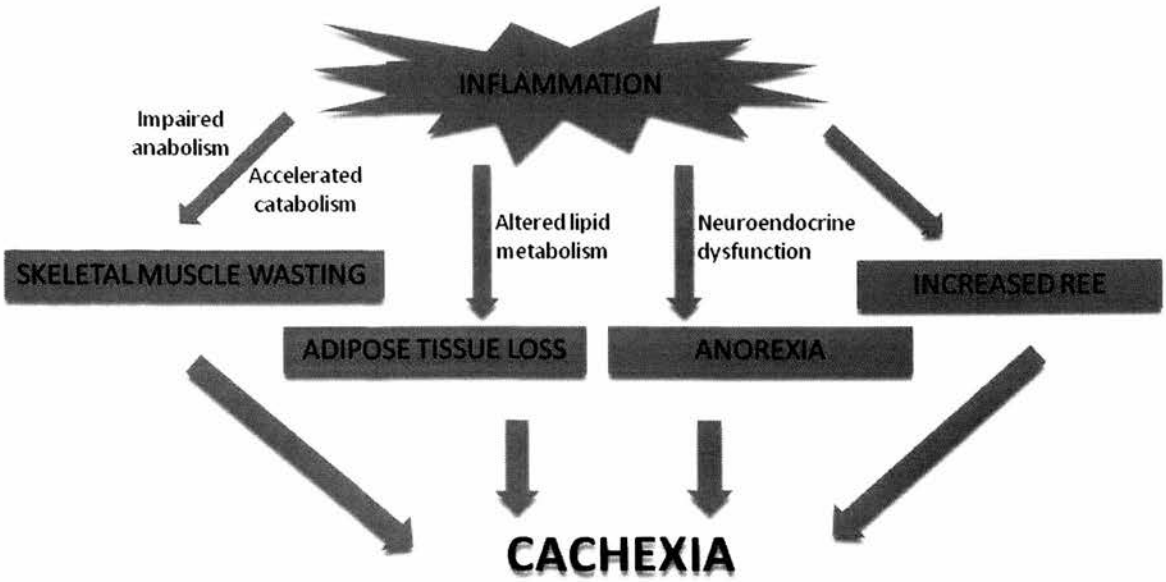
The economic costs of cachexia extend much further than the costs of therapeutic diets, nutritional supplements, medications, laboratory tests, and supplies. Staff salaries, service costs, and other indirect medical costs related to the provision of medical care must also be included. Unfortunately, it is difficult to assess accurately the financial costs of cancer cachexia due to paucity of data. The extent of the problem can, however, be highlighted by examining the overall costs of cancer treatment (around $190 billion in the United States in 2007).

Less apparent are the costs associated with managing the consequences of involuntary weight loss. Involuntary weight loss is associated with anaemia, postural hypotension, cognitive dysfunction (i.e., confusion and impaired cognition), falls, and hip fractures (Knudtson *et al*, 2005). Pressure ulcers are another frequent complication seen in patients near the end of the 'cachexia journey' (Collins, 2003). Currently, billions of dollars are spent each year to treat these complications. For example, the total direct cost of falls is approximately $20 billion and the estimated cost of pressure ulcer management is currently about $7 billion annually (Kumar *et al*, 2004; Stevens *et al*, 2006).

1.4.2 Mechanisms of cancer cachexia

Cachexia is brought about by a synergistic combination of a dramatic decrease in appetite and an increase in metabolism of fat and lean body mass. Although the specific molecular mechanisms leading to the development of cachexia are not fully understood, systemic inflammation appears to play a central role (Morley *et al*, 2006) (Figure 1.4). Inflammation results in a number of metabolic changes that are often characterized by negative energy balance, increased thermogenesis and anorexia. These responses are mediated by cytokines (Argiles *et al*, 2009; Dantzer & Kelley, 2007).

Figure 1.4 Proposed mechanisms implicated in cachexia



Anorexia

Cytokines produced during inflammation can affect the brain by several pathways: i) they can act on peripheral tissues producing hormones such as leptin which influence the activity of the brain; ii) they can act on peripheral neurons which project to the brain through the vagus nerve; iii) they can also directly enter the brain and act locally (Guyon *et al*, 2008).

Many cytokines are known to have an effect on appetite, including interleukin (IL)-1α, IL-1β, and IL-6 as well as tumour necrosis factor- α (TNF-α) (Plata-Salaman *et al*, 1988). IL-1, in

particular, has been clearly associated with the induction of anorexia (Plata-Salaman, 2000), by blocking neuropeptide Y (NPY)-induced feeding. The levels of this molecule (a feeding-stimulating peptide) are reduced in anorectic tumour-bearing rats and a correlation between food intake and brain IL-1 has been found in anorectic rats with cancer (Chance *et al*, 1994).

Increased energy expenditure

Resting energy expenditure (REE) is significantly higher in those with an elevated acute phase response (APR) (Falconer *et al*, 1994). The APR is a series of changes in liver protein synthesis, which shifts from production of albumin to acute phase proteins (APP), such as C-reactive protein (CRP), fibrinogen, serum amyloid A, and α-1 antitrypsin, in response to an insult such as inflammation.

There is a known association between the development of an APR and the rate of loss of body mass in lung and gastrointestinal cancers (McMillan *et al*, 1998), and in patients with non- small cell lung cancer (NSCLC), serum levels of IL-6 were found to correlate with concentrations of circulating CRP (McKeown *et al*, 2004).

Altered carbohydrate metabolism

Tumour growth results in energetic consequences which locally changes from aerobic to anaerobic dissimilation and causes metabolic adaptation in the liver. Tumours demonstrate changed metabolic profiles with hypoxia in the growing tumour (Harris, 2002) and increases in glucose uptake (Jerusalem *et al*, 2002), glycolysis and lactic acid production.

From the moment tumour cells start the anaerobic dissimilation the increasing glucose demand can only be met by accelerated hepatic gluconeogenesis, characterised by increased activity of the Cori cycle (Holroyde *et al*, 1984). This cycle consists of the

fermentation of glucose to lactic acid in the tissues, and the conversion of lactic acid to glucose in the liver (Roh *et al*, 1984).

The increased hepatic glucose production is also partially due to a lack of inhibition of gluconeogenesis by insulin (Yoshikawa *et al*, 1999). Resistance to insulin occurs in patients with cancer and is negatively correlated with the APR, suggesting the involvement of inflammatory reactions (Yoshikawa *et al*, 2001).

Altered lipid metabolism

The multifunctional cytokine, TNF-α exerts a series of biological actions in different cells, tissues, organs, and species and has been demonstrated to regulate and interfere with energy metabolism, especially lipid homeostasis. A large body of research suggest that the effects of TNF-α on lipid metabolism mainly include five aspects: i) suppression of free fatty acid (FFA) uptake and promotion of lipogenesis; ii) induction of lipolysis; iii) inhibition of lipid-metabolism-related enzyme activity; iv) regulation of cholesterol metabolism; v) regulation of other adipocyte-derived adipokines (Chen *et al*, 2009).

While TNF-α is the first cytokine reported to have catabolic effects on fat cells, subsequent studies have shown that multiple cytokines acting through multiple receptors have similar activities. TNF-β, interferon-α, interferon-γ, IL-1, and IL-6 decrease lipoprotein lipase activity and increase lipolysis in cultured fat cells (Doerrler *et al*, 1994). TNF-β and both interferons also decrease de novo fatty acid synthesis in cultured fat cells (Doerrler *et al*, 1994). The catabolic effects of cytokines on adipose tissue triglyceride metabolism is also a possible contributor to the development of cachexia (Tisdale, 2009).

Impaired protein synthesis

Inflammatory-mediated signalling may limit muscle protein synthesis by several mechanisms. Inflammatory cytokines such as TNF-α and IL-6 are likely to play an important role. TNF-α can activate the transcription factor NF-kB, which inhibits the synthesis of the muscle specific transcription factor MyoD, thereby inhibiting differentiation (Acharyya & Guttridge, 2007). In addition, a recent study found that skeletal muscle protein synthesis was dramatically reduced by relatively low-dose IL-6 infusion in humans (van Hall *et al*, 2008).

Preliminary reports have described marked elevations in myostatin (a negative regulator of muscle mass that inhibits myogenic proliferation and differentiation) in cachectic cancer patients. Animal models suggest that the inflammatory cytokine TNF-α is at least partially responsible for this increase (Costelli *et al*, 2008). TNF-α is also known to negatively influence the anabolic mTOR signaling pathway, which is a major mediator of anabolic responses in skeletal muscle (Lang *et al*, 2007).

IL-15 is a cytokine which is highly expressed in skeletal muscle (Grabstein *et al*, 1994). IL-15 is anabolic in nature and acts by activation of protein synthesis and concomitant inhibition of protein degradation in cultured skeletal muscle cells (Quinn *et al*, 1995). IL-15 administration in an *in vivo* rodent model of cancer cachexia has been shown to inhibit skeletal muscle wasting (Carbo *et al*, 2000). Muscle and serum IL-15 levels are thought to be reduced in cachexia (Pajak *et al*, 2008).

Increased protein degradation

Inflammatory stimulation activates pathways associated with muscle protein breakdown (Burckart *et al*, 2010). In cancer cachexia, the ubiquitin proteasome pathway is greatly influenced (Lecker *et al*, 1999). Many of these effects are thought to occur through activation

of NF-kB by upstream factors such as TNF-α (Ladner *et al*, 2003). NF-kB stimulates transcription of the ubiquitin E3 ligase muscle ring finger (MuRF)-1, which is known to positively regulate activity of the ubiquitin proteasome pathway (Cai *et al*, 2004).

1.5 Biomarkers of cancer cachexia

Cachexia in its advanced phase (where patients may have lost 20-30% of their body weight) is easily identified. However, at this stage, the primary initiating events are frequently compounded by secondary factors (e.g. prolonged patient bed rest), and it is often impossible to attempt any realistic form of intervention, either practical or (given the patient's almost imminent demise) ethically advisable. One systematic approach to the treatment of cachexia requires early identification of patients at risk of cachexia and the institution of prophylactic measures to attenuate its progression. The development of predictive or early biomarkers of cachexia is thus essential to aid in the selection of patients for early management.

The search for potential biomarkers of cachexia has encompassed several different body compartments including plasma, urine, tumour and skeletal muscle. More recently, the search has focused on the patients' genome.

1.6 Rationale of genetic involvement in cancer cachexia

Based on current knowledge of demographic and clinical factors, it is not possible to predict, for any given cohort of patients, who will develop cancer cachexia and who will not. It is also not possible to predict accurately who will develop cachexia quickly versus those who may develop the syndrome at a slower pace. Such variation may, in part, be due to the patient's genotype. The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome (e.g. systemic inflammation). However, it must be said that, unlike other common diseases where twin studies have suggested a clear heritable component to the disease (e.g. Crohn's disease, etc), there have been no such studies to support a heritable component to cachexia so far.

1.7 Genetic approaches to study cancer cachexia

One of the most common approaches to find genes involved in complex traits are genetic association studies. The rationale of genetic association studies is to detect association between one or more genetic polymorphism(s) and a trait or disease. Association studies identify polymorphisms in which an allele occurring in the general population occurs at a different frequency in the disease group. In these instances, the disease associated allele does not cause the disease in the same way that a Mendelian mutation does but increases susceptibility to the disease as a genetic risk factor, most likely in conjunction with other genetic and/or environmental risk factors. Association studies can either be direct or indirect. In direct association studies, target polymorphisms which are themselves putative functional variants (for example a SNP variant in a gene at a codon that changes an amino acid) are

genotyped in both the general (control) and also trait (disease) population. A statistically different frequency of the alleles and/or genotypes in the control population versus the disease group would suggest that the polymorphism in question has a direct effect on disease pathogenesis. However, it is likely that many causal variants contributing to complex disorders will be non-coding. These variants could include those that affect gene regulation, expression or alternative splicing and such functional variants are difficult to predict. For this reason, most association studies are indirect; where the polymorphisms genotyped in the control populations and trait populations are surrogates for the unknown causal locus.

Identifying susceptibility genes for complex disorders by the indirect method depends on the existence of an association between the causal variants and surrounding polymorphisms nearby. This association is termed linkage disequilibrium (LD) and is defined as the non-random association of alleles at two or more loci and describes a situation in which correlation between nearby variants such that the alleles at neighbouring markers (observed on the same chromosome) are associated within a population more than if they were expected by chance.

1.7.1 Candidate gene association analysis in cachexia

The finding that only a proportion of patients with chronic disease develop cachexia has prompted studies looking for genetic polymorphisms that may underlie differential susceptibility (Tan *et al*, 2008). The most frequent targets for these studies have been genes encoding pro-inflammatory cytokines, such as TNF-α and some of the interleukins. This is based on the hypothesis that continued systemic inflammation plays a central role to the pathogenesis of cachexia (see above).

One of the earliest genetic susceptibility studies by Broekhuizen *et al.* (2005) found that a -511 polymorphism in the *IL1B* gene (rs16944) correlated strongly with cachexia in patients with COPD. The C/C genotype at this position in the gene was associated with frequent cachexia, while the T/T genotype was not associated with cachexia.

While this finding has not been confirmed in subsequent studies, Zhang *et al.* (2007) have found that a separate polymorphism within the IL1B gene is associated with cachexia. Gastric cancer patients in China with cachexia showed a significantly higher prevalence of the (rs1143634) *IL1B* +3954 T allele than those without, and the *IL1B* +3954 C/T genotype was associated with an 2.5 times increased risk of developing cachexia. Unfortunately, the reverse association was found in another study involving patients with gastric and gastro-oesophageal junction cancer (Jatoi *et al*, 2007). Patients with the *IL1B* +3954 C/T and T/T genotypes showed greater improvements in their weight and in survival over time than did patients with the C/C genotype.

A recent article has recently investigated the relationship between five cytokine polymorphisms and markers of nutritional status among patients with gastro-oesophageal cancer (Deans *et al*, 2009a). Possession of the (rs1800896) *IL10* -1082 G allele was found to be associated with increased weight loss and the G/G genotype was associated with a 2.3 times increased risk of developing cachexia. A separate study involving patients with gastric cancer in China confirmed that the G allele was more prevalent in patients with cachexia (Sun *et al*, 2010). The study also found that individuals with the (rs1800871) *IL10* -819 C/C genotype were at increased risk of developing cachexia.

A further two cytokine polymorphisms have been associated with cachexia. The (rs1800796) *IL6* -634 G allele has been shown to be significantly more prevalent in Chinese pancreatic cancer patients with cachexia compared with those without cachexia (Zhang *et al*, 2008) (Table 1). In patients with gastric cancer in China, the frequency of (rs2227306) *IL8* +781 T

allele was noted to be significantly increased in patients with cachexia and the +781 T/T genotype was observed to be associated with a significantly increased risk of cachexia (Song *et al*, 2009).

While much attention has been placed on cytokine polymorphisms and their association with cachexia, it is of note that mechanisms surrounding the pathogenesis of cachexia is complex and is likely to involve gene products which act both upstream and downstream of cytokines. Pathways not affected by cytokines may also play a role in the development of cachexia. It is therefore highly likely that genetic variations in non-cytokine genes may also contribute to the susceptibility of developing cachexia. For example, a study by Vigano *et al.* (2009) have shown that the Angiotensin converting enzyme gene (*ACE*) insertion/deletion polymorphism (rs4646994) is associated with lower total fat mass and lean body mass in patients with gastro-intestinal cancer and non-small cell lung cancer.

1.8 Hypothesis

1. Altered body composition in advanced cancer patients carries prognostic significance
2. Sarcopenic obesity is an identifiable entity that relates to shortened survival
3. Genetic variation contributes to the prevalence of cancer cachexia
4. Genetic variance in non-cytokine genes may also contribute to the susceptibility of developing cancer cachexia

1.9 Aims of thesis

1. To evaluate body composition in advanced cancer patients using image analysis of CT scans and relate changes to outcomes
2. To evaluate specifically the presence of sarcopenic obesity in advanced cancer patients and relate it to outcomes
3. To undertake a systematic review to explore genetic polymorphisms in potential candidate genes involved in the development of cancer cachexia
4. To identify other possible candidate genes of cachexia by utilising mRNA microarray technology to analyse the differential expression of genes in skeletal muscle in patients with and without cachexia.
5. To undertake genetic association studies assessing for susceptibility to cachexia as informed by the results of the systematic review and microarray study.

1.10 Plan of thesis

The prominent clinical feature of cachexia has traditionally been understood to be weight loss and the relationship between changes in total body weight over time and cancer outcomes has a long history.

It is widely accepted that changes in body weight may involve any of its major tissue compartments (lean tissues, adipose tissues, and bone) and that within these compartments; there may be specific changes of organs, tissues, and regions.

A feature of body composition changes such as differential accumulation of lipids in different compartments and tissues is that these changes are invisible. Skeletal muscle wasting is another phenomenon, which can be hidden within the bulk of body weight and body weight change, and there is a new recognition of sarcopenia (severe muscle wasting) as a clinically important phenomenon. Sarcopenia is a term denoting a reduced quantity of skeletal muscle more than 2 SDs below that typical of healthy adults (Baumgartner *et al*, 1998).

The aging process is often paralleled by decreases in muscle and increases in fat mass, which may culminate in sarcopenic obesity (Roubenoff, 2000; Zamboni *et al*, 2008); this condition combines the health risks and functional losses of both conditions and is increasingly prevalent in Westernized countries (Bouchard *et al*, 2009; Rolland *et al*, 2009).

It is important to note that a unit of human body weight, or of body weight change, does not have a constant composition and the recently published consensus definition of cancer cachexia (Evans *et al*, 2008) notably makes a distinction between the behaviour of skeletal muscle and adipose tissue: 'cachexia, is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. This acknowledgement of a distinction between the behaviour of muscles and adipose tissues, bring the need for specific body composition measures clearly into view.

Pancreatic cancer has been noted previously to be a useful model for the study of cancer cachexia (Fearon *et al*, 1999). By utilising a novel method of analysis enabling routine derivation of body composition data from computed tomography scans, Chapter 3 assesses if measures of body composition, specifically skeletal muscle, have any prognostic value in

patients with pancreatic cancer. Time course changes in regional body fat and lean tissue compartments in pancreatic cancer patients were also studied.

In the current chapter, the rationale and background behind the genetic association to cancer cachexia was reviewed. At the onset of this work, support for a heritable component to the susceptibility of developing cachexia has been highlighted in a few studies mainly focussed on variants in inflammatory cytokine genes. However, there is a distinct lack of association studies on genes regulating other key mechanisms that underlie the cachexia syndrome such as muscle and adipose tissue metabolism.

A systematic literature review is presented in Chapter 4 which explores genetic polymorphisms with known functional or clinical significance in potential candidate genes involved in the development of cancer cachexia. It also identifies the polymorphisms with the most likely potential as susceptibility markers for cancer cachexia.

Chapter 5 explores further possible candidate genes involved in the development of cachexia by examining the differential expression of genes in muscle of cachetic versus non-cachetic patients using mRNA microarray technology.

A large scale candidate gene association study is presented in Chapter 6. Utilising data from the systemic literature review and the microarray study, 129 SNPs across 80 genes were analysed in 775 cancer patients assessing their contribution to cachexia susceptibility. A further validation association study was also performed on an independently recruited cohort (n=101) of cancer patients

In chapter 7, the implications of the data presented throughout the thesis are discussed. The results are placed in the context of current work performed during and after the completion of the experimental work of the thesis. A plan for continuation of the work is also discussed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study participants

2.1.1 Body composition study (Chapter 3)

This was a retrospective cohort study. All patients referred to the regional cancer centre in Edmonton, Alberta, Canada between January 2004 and October 2008 were considered for the study. Patients with a primary diagnosis of pancreatic cancer entering a palliative programme who had an abdominal CT scan within 60 days of initial assessment were selected for the study. Patients with ampullary carcinoma, cholangiocarcinoma or neuro-endocrine tumours were excluded. Coding of the primary cancer by its site and morphology, clinical information, and demographic information were obtained from the Alberta Cancer Registry for every patient in the cohort. The Alberta Cancer Registry is a computerised database of all cancer cases in the region (population 1.8 million).

2.1.2 Microarray muscle gene expression study (Chapter 5)

Patients were recruited to this study between August 2007 and October 2008. All patients were above 18 years of age had a diagnosis of upper gastro-intestinal cancer (oesophageal, gastric, pancreatic) and were undergoing surgery with the intent of resection of the primary tumour. All patients were enrolled before any treatment was initiated and within 1-2 weeks of diagnosis. Newly diagnosed patients were identified principally from the regional oesophago-gastric and hepatobiliary cancer multidisciplinary team (MDT) meetings at the Royal Infirmary of Edinburgh, UK. The oesophago-gastric and hepatobiliary MDTs provides regional referral services for patients with oesophageal, gastric and pancreatic cancer from the Lothian and Borders regions. Patients with newly diagnosed upper gastro-intestinal cancer within these regions are referred to the MDTs which meet on a weekly basis to discuss all new referrals and to decide on individual patient management strategies.

Participants were recruited at their surgical clinic appointments at the Royal Infirmary of Edinburgh.

Control patients (i.e. with benign conditions necessitating abdominal surgery e.g. incisional hernias) were recruited at outpatient surgical clinics prior to their surgery.

2.1.3 Candidate gene association study (Chapter 6)

Patients were recruited from three centres for this study with a separate independent validation cohort of patients recruited from a fourth centre. Exclusion criteria were as follows: i) under 18 years of age; ii) learning disability, and mental health problems; iii) inability to give written, informed consent.

The main study cohort was recruited from this study between January 2004 and November 2008 from the following three institutions:

1) NHS Lothian, Edinburgh, UK
2) Cross Cancer Institute, Edmonton, Canada
3) McGill Cancer Center, Montreal, Canada

NHS Lothian

All patients recruited had either a confirmed diagnosis of gastro-oesophageal or pancreatic malignancy. Approximately 300 retrospective patients recruited for previous studies with blood stored in a tissue bank with relevant weight loss data were entered into the study. Another 150 patients were recruited prospectively. These patients were identified principally from the regional oesophago-gastric and hepatobiliary cancer multidisciplinary team (MDT)

meetings at the Royal Infirmary of Edinburgh, UK. Patients were recruited at first presentation to surgical or oncological clinic at Royal Infirmary of Edinburgh or Western General Hospital, Edinburgh, UK.

Cross Cancer Institute

Approximately 190 patients were entered into the study. All patients had been previously recruited for clinical research studies with relevant weight loss data and had blood stored in a tissue bank. All patients had a confirmed diagnosis of non-small cell lung cancer (NSCLC).

McGill Cancer Center

All patients recruited had confirmed diagnosis of colorectal, pancreatic or non-small cell lung cancers. Patients had previously participated in clinical research studies with relevant weight loss data and had blood stored in a tissue back. Approximately 130 patients were entered into the study.

Validation cohort

Patients for the validation cohort were recruited from Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland from January 2007 to December 2008. Patients with all proven malignancies were considered and 101 patients were prospectively recruited.

2.2 *Clinical and pathological data collection*

Demographic data was recorded and included age and sex for each patient across all centres. The date of diagnosis was also recorded in all instances.

Histological confirmation of disease was obtained where possible and patients were staged according to the American Joint Committee on Cancer stage groupings (AJCC). Tumours of the gastro-oesophageal junction were classified according to Siewert and Stein and those classified as type I and II were staged as oesophageal tumours and type III as gastric cancers.

2.3 *Nutritional assessment*

All patients underwent measurements of height and weight at the time of recruitment. Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. There is evidence to support the reliability of self-reported weight and weight history (Perry *et al*, 1995; Stunkard & Albaum, 1981). Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost. Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2).

2.4 Collection of biological samples

2.4.1 Blood collection

Patients recruited for the gene microarray and candidate gene studies had up to 10ml of blood collected into EDTA tubes and tubes with gel separator and clot activator. The blood was usually collected when the patients were having blood taken as part of their on-going clinical care as requested by their supervising clinician and not merely for participation in the research study.

2.4.2 Skeletal muscle biopsies

For patients recruited for the gene microarray study, biopsies were taken at the start of open abdominal surgery. The edge of the *rectus abdominis* was exposed and a $1cm^3$ biopsy was removed with sharp dissection. Excess blood was removed and the biopsy was snap frozen immediately in liquid nitrogen and stored at -80 degrees until further analysis.

2.5 Ethical approval

All studies contained in this thesis received full ethical permission from their respective host institutions. Ethical approval for research carried out in Edinburgh was granted by the Lothian Research Ethics Committee (reference 08/S1103/19).

2.6 Blood assays

2.6.1 Measurement of C-reactive protein (CRP)

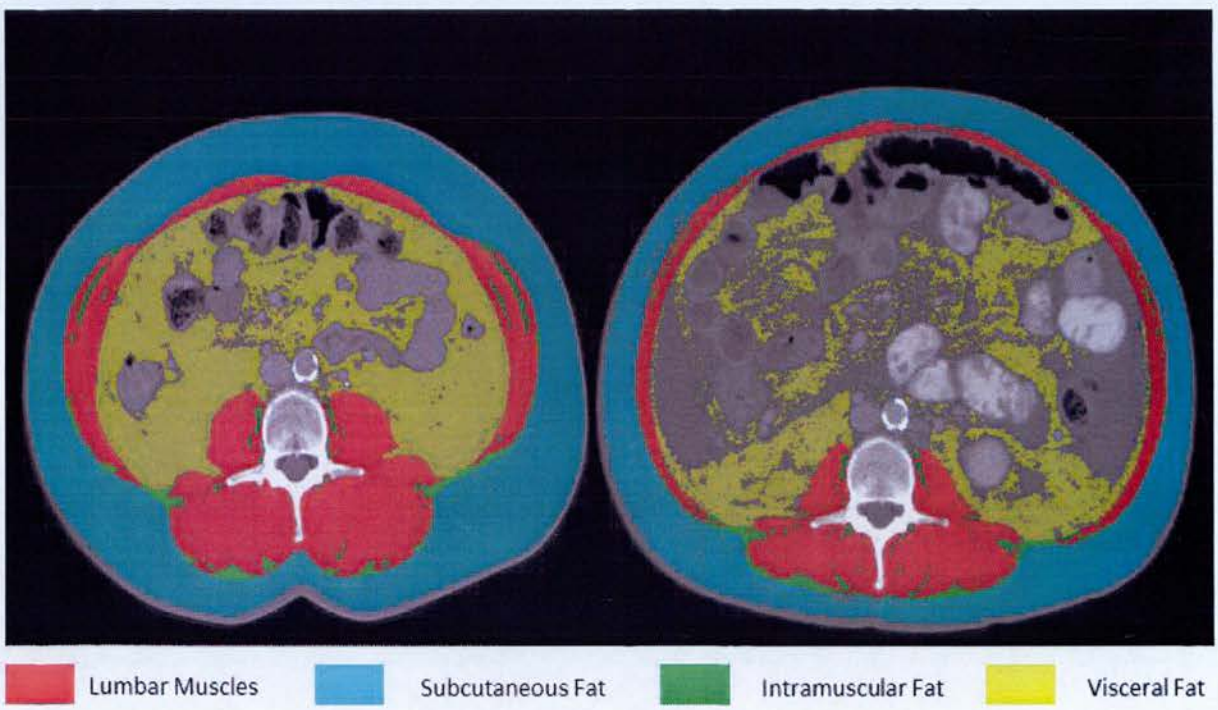
Serum CRP concentration was measured using an automated immuno-turbidimetric assay. Using this assay, a concentration of 10mg/l represents the upper limit of normal range with most healthy individuals having a serum concentration <2mg/l.

2.7 Body composition analysis

2.7.1 Estimation of total body skeletal and adipose tissue using computed tomography (CT) scans

CT scans used for analysis were performed solely for routine cancer care. Two consecutive transverse CT images extending from the third lumbar vertebrae (L3) in the inferior direction were assessed for each scan date and then averaged; the foremost image being the one in which both transverse processes were first clearly visible. Images were analyzed with SliceOmatic V4.3 software (Tomovision, Montreal, Canada) which enables specific tissue demarcation using Hounsfield unit (HU) thresholds. Skeletal muscle was identified and quantified by HU thresholds of -29 to +150 (Mitsiopoulos *et al*, 1998). The muscles in the L3 region contain *psoas, erector spinae, quadratus lumborum, transversus abdominus, external and internal obliques, and rectus abdominus.* The following HU thresholds were used for adipose tissues: -190 to -30 for subcutaneous and intramuscular adipose (Kvist *et al*, 1986), and -150 to -50 for visceral adipose (Vehmas *et al*, 1996). Tissue boundaries were manually corrected as needed (Figure 2.1).

Figure 2.1 Cross sectional CT images at L3 showing demarcation of skeletal muscle and adipose tissue using Slice-o-matic software



Cross-sectional areas (cm^2) were computed automatically by summing tissue pixels and multiplying by pixel surface area. All CT images were analysed by a single trained observer. Cross-sectional area for muscle and adipose tissue was normalised for stature (cm^2/m^2) and reported.

Routine diagnostic CT scans usually only evaluate the chest, abdomen and pelvis and therefore only partial images are available to determine skeletal muscle mass. Estimates of whole body stores were generated from the raw data (cm^2) using the following regression equations by Mourtzakis *et al.* (2008) which show a close correlation between muscle and

fat areas in CT images at the 3rd lumbar vertebrae and whole body compartments of fat-free mass and fat mass respectively.

- Total body fat-free mass (FFM) (kg) = 0.3 x [skeletal muscle at L3 (cm^2)] + 6.06, (r = 0.94)
- Total body fat mass (FM) (kg) = 0.042 x [total adipose tissue at L3 (cm^2)] + 11.2, (r = 0.88)

2.8 Cellular methods

2.8.1 Nuclear protein extraction and RNA isolation from muscle

Approximately 20 mg of muscle was re-suspended in 180 µl of low salt lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, protease inhibitors (Roche Diagnostics; 1 tablet per 10 ml)) and ground using a handheld homogenizer. Samples were incubated on ice for 5 minutes before two cycles of freeze-thaw lysis. After a brief vortex, samples were centrifuged at 4,000 rpm for 3 minutes. The supernatant was removed and the pellet (containing the nuclei) re-suspended in 40 µl high salt extraction buffer (20 mM HEPES, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, protease inhibitors (Roche Diagnostics; 1 tablet per 10 ml)). Samples were incubated on ice for 30 minutes with gentle mixing of the tubes every 5 to 10 minutes. Samples were centrifuged at 4,000 rpm for 5 minutes at 4°C. The supernatant which now contained the nuclear proteins was aliquoted into tubes and stored at -80°C.

Total RNA was extracted from the supernatant using TRIzol (Invitrogen, Paisley, UK) reagent. 800 µl of TRIzol reagent was added to each sample. Samples were incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. 160 µl of chloroform was then added. Tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube and RNA was precipitated by mixing with 400 µl of isopropyl alcohol. Samples were incubated at 15 to 30°C for 10 minutes and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was then removed and the RNA pellet washed with 800 µl 75% ethanol. The sample was mixed by vortexing and then centrifuged at 7,500g for 5 minutes at 4°C. The RNA pellet was then air dried for 5 to 10 minutes and then re-suspended in DEPC treated water.

2.8.2 DNA extraction from blood

Genomic DNA was extracted from samples of blood taken in EDTA tubes. The Wizard Genomic DNA purification kit (Promega, Southampton, U.K) was used. Whole blood was centrifuged at 1,500g for 5 minutes at 4°C. 300 µl of the cellular layer was added to 900 µl of cell lysis solution in a 1.5 ml micro centrifuge tube and mixed thoroughly by inverting the tubes several times. The mixture was incubated for 10 minutes at room temperature followed by centrifugation at 15,000g for 20 seconds also at room temperature. The supernatant was discarded and the process of adding cell lysis solution, incubation and centrifugation was repeated once more. 300 µl of nuclei lysis solution was added to the pellet and the tube was vortexed to resuspend the white cells. The solution was pipetted gently several times to lyse the white cells. 1.5 µl of RNase solution (Promega) was added to each tube, mixed and

incubated at 37°C for 15 minutes. After allowing the tubes to cool to room temperature, 100 µl of protein precipitation solution was added and mixed by vortex for 15 seconds. Samples were then centrifuged at 15,000g for 3 minutes at room temperature. The supernatants were then transferred to a clean 1.5 ml micro centrifuge tube and mixed with 300 µl of pure isopropanol. Gentle mixing reveals the appearance of the DNA as white thread-like strands. Further centrifugation at 15,000g for 1 minute at room temperature allows the DNA to be collected as a pellet at the bottom of the tube. The supernatant was then decanted and 300 µl of ethanol 70% is added to wash the pellet. The samples were then centrifuged again for a further minute at 15,000g and the ethanol carefully aspirated away. 100 µl of DNA rehydration solution (10 mM Tris-HCl and 1 mM EDTA) was added to each tube and samples were incubated at 65°C for 1 hour. DNA samples were then stored at 4°C.

2.8.3 Quantification of RNA and DNA

DNA and RNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer. Absorbance at 260nm were used for quantification of nucleic acids, optical density of 1 corresponding to 50ng/µl DNA and 40ng/µl RNA. Absorbance ratios (260 nm/280 nm) of approximately 1.8 for DNA and 2.0 for RNA indicated that the nucleic acid preparations were sufficiently free from protein contamination for downstream experiments.

2.9 Gene expression analysis

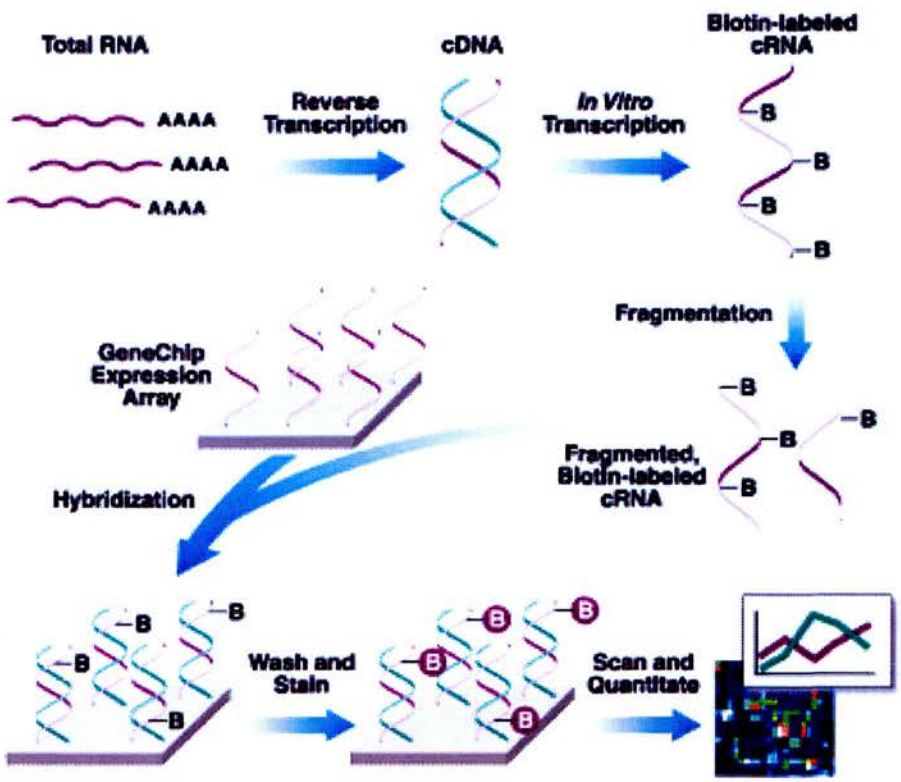
The Affymetrix GeneChip short-oligonucleotide arrays (Affymetrix, High Wycombe, U.K) was utilised for muscle gene expression analysis. These arrays are small wafers of which the area is divided into thousands of very small pieces (spots). Probes are chosen to fit to a

unique part of the target RNA of which the quantity has to be measured. They are complementary to this sequence since the RNA will then automatically bind (hybridize) to the probe. The target RNA is labelled with a fluorescent label. Finally, the RNA molecules that did not bind to any probe are washed away from the array and the amount of fluorescence for each probe is measured in a scanner. This amount represents the amount of RNA that was present for each gene. The Affymetrix technology uses multiple probes per gene, the probe-set, to measure RNA abundances, with up to 1.3 million probe-sets on a single array.

The outline of the Affymetrix Genechip analysis protocol is illustrated in figure 2.2. The genechips were scanned using the GeneChip scanner 3000 (Affymetrix, High Wycombe, U.K).

Figure 2.2 Outline of Affymetrix Genechip analysis protocol

1. Total mature RNA is isolated from tissue/cell being studied.
2. RNA is reverse transcribed into cDNA for storage purposes
3. Prior to running the array, cDNA is transcribed back to cRNA and labeled with Biotin.
4. Labeled cRNA is randomly fragmented to between 30 to 400 base pairs in length
5. Fragmented, Biotin-labeled cRNA is added to the Genechip array
6. cRNA is hybridized to complimentary probes in the array.
7. cRNA is tagged with florescent label and array is washed to remove any non-hybridized RNA
8. Lastly, the array is scanned for quantitative analysis of RNA present for each gene.



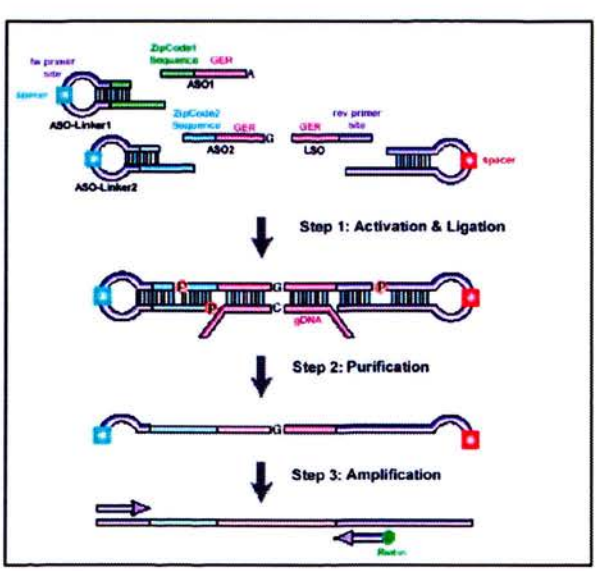
2.10 Single nucleotide polymorphism (SNP) genotyping

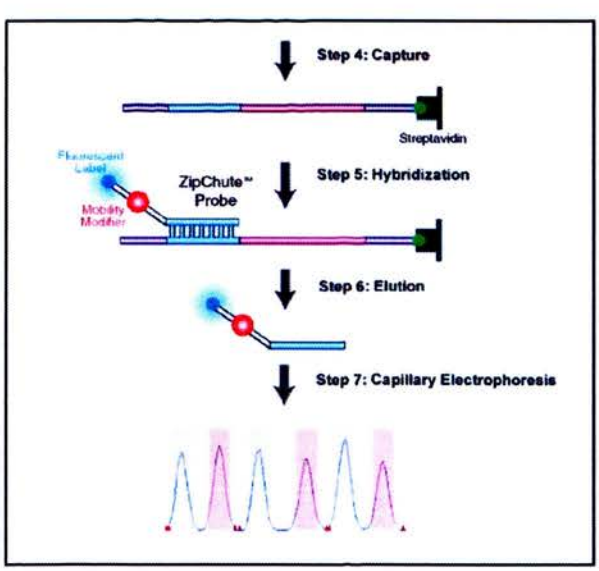
The SNPlex Genotyping System (Applied Biosystems, California, USA) was utilised for SNP genotyping. The SNPlex Genotyping System utilises a suite of pre-optimized universal assay reagents kits and a set of SNP-specific ligation probes to perform genotyping up to a 48-plex level (i.e., 48 SNPs genotyped in a single reaction). The SNPlex Genotyping System is based on the oligonucleotide ligation/PCR assay (OLA/PCR) with a universal ZipChute™

probe detection for high throughput SNP genotyping. Fluorescently labelled ZipChute probes are hybridized to complementary ZipCode™ sequences that are part of genotype specific amplicons. These ZipChute probes are eluted and detected by electrophoretic separation on a 3730 DNA Genetic Analyzer (Applied Biosystems, California, USA). The assay workflow for the SNPlex Genotyping System is illustrated in figure 2.3

Figure 2.3 Workflow for SNPlex Genotyping System

1. Allele-specific oligonucleotide ligation (OLA) reaction; during which allele-specific oligonucleotide (ASO) probes and locus-specific oligonucleotide (LSO) probes hybridize to the genomic target sequence. A unique ZipCode sequence is attached at the 5′ end of the genomic equivalent sequence within each ASO.
2. Purification of OLA reaction by exonucleolytic digestion of excess probes and linkers.
3. Universal PCR reaction to amplify ligation products, and biotinvlation.
4. Capturing of biotin-labelled PCR products in streptavidin coated microtiter plates.
5. Binding of fluorescently labelled ZipChute probes to single-strand PCR products; Each ZipChute probe contains a sequence complementary to the unique ZipCode sequence within each ASO.
6. Elution of hybridized ZipChute probes; and
7. Detection by Capillary Electrophoresis





The Applied Biosystems' GeneMapper® Software (version 4.0) was used to perform automated allele calls and genotype clustering of each individual sample. Any SNPs with less than 90% of the sample auto-called by the software were either rescored manually or discarded if clustering confidence was low.

2.11 Statistics

2.11.1 General statistics

General statistical analyses were performed using SPSS® Version 15.0 (SPSS, Chicago, Illinois, USA). Data are presented as mean ± standard deviation unless otherwise stated. Univariate and multivariate survival analysis and calculation of hazard ratios in Chapter 3 were performed using a Cox regression model. Comparisons between groups of patients were assessed using one way ANOVA or Pearson's chi-square test. Survival curves were constructed using the Kaplain-Meier technique. Log-rank test was used to compare survival between groups of patients. Comparison of data at different time points for body composition analysis in Chapter 3 was performed using the paired t-test. *P* values <0.05 were regarded as statistically significant.

2.11.2 Microarray statistical analysis

Microarray data were analysed using the Microarray Suite software (MAS) version 5.0 (Affymetrix). Genes called absent on every array by the MAS5 software were filtered from the data and remaining genes analysed using the significance analysis of microarrays (SAM) and Limma packages in the Bioconductor suite. Genes covarying with weight loss were identified by a false discovery rate (FDR) of <10%.

2.11.3 SNP genotyping statistical analysis

Statistical analyses were performed using PLINK (version 1.06). Unconditional logistic regression was employed to calculate odds ratios (OR) and their 95% confidence intervals (95% CI) for the minor allele of individual SNPs and its association with each proposed cachexia phenotype. To account for multiple testing, permutation testing was performed by running the adaptive permutation test in PLINK within each proposed phenotype.

CHAPTER 3

BODY COMPOSITION IN CANCER CACHEXIA: PANCREATIC CANCER

3.1 Summary

Aims

Using pancreatic cancer as a model for cancer cachexia, the aim of this chapter is to evaluate if weight and body composition, specifically sarcopenia, assessed from diagnostic computed tomography (CT) scans, is of prognostic value. The nature and extent of tissue loss over subsequent months was also evaluated.

Methods

111 pancreatic cancer patients entering a palliative therapy programme, who had CT images and undergone nutritional screening, were studied. In patients where follow-up scans were available (n=44), longitudinal changes in body composition were studied at a mean of 230 ± 62 and 95 ± 60 days prior to demise.

Results

62 patients (55.9%) were sarcopenic, 44 (39.6%) were overweight/obese, and 18 (16.2%) were both. Age ≥59 years (HR 1.71, 95%CI 1.10 – 2.66, p=0.018), and overweight/obese sarcopenia (HR 2.07, 95%CI 1.23 – 3.50, p=0.006) were identified as independent predictors of survival on multivariate analysis. Longitudinal analysis revealed that total fat-free mass (FFM) index decreased from 15.5 ± 2.5kg/m^2 to 14.5 ± 2.0kg/m^2 (p=0.002), and total fat mass (FM) index decreased from 7.5 ± 2.0kg/m^2 to 6.0 ± 1.5kg/m^2 (p<0.0001) over 135 days.

Conclusions

Sarcopenia in overweight/obese patients with advanced pancreatic cancer is an occult condition but can be identified using CT scans. This condition is an independent adverse prognostic indicator. Longitudinal analysis of body composition in pancreatic cancer patients revealed a loss of both skeletal muscle and adipose tissue in the course of the cancer journey but, adipose tissue was lost more rapidly than muscle.

3.2 Introduction

One of the most distressing features of pancreatic cancer is marked and progressive weight loss. Cachexia occurs in up to 80% of deaths in patients with advanced pancreatic cancer (Dewys *et al*, 1980). Pancreatic cancer has been shown previously to be a good model for the study for cancer cachexia (Fearon *et al*, 1999).

Due to the epidemic of obesity in Western Society, a substantial proportion of oncology patients at the start of palliative therapy now have a BMI in the overweight range (Irigaray *et al*, 2007) and this can confound conventional measures used for risk stratification. Indeed recent studies have reported that obesity (i.e. BMI ≥30 kg/m^2) in the presence of sarcopenia is predictive of morbidity and mortality in both malignant and non-malignant disease.(Honda *et al*, 2007; Prado *et al*, 2008) The development of novel methods of image analysis enabling routine derivation of body composition data from diagnostic CT scans (and in particular the estimation of skeletal muscle mass) provides an opportunity to assess if measures of body composition has any prognostic value in patients with pancreatic cancer . The present study focused on sarcopenia specifically, both in the presence or absence of an elevated BMI.

When considering the significance of sarcopenia in a given population, it is important to know the likely longitudinal pattern of wasting and how this may be altered by concomitant systemic oncological therapy. The nutritional and metabolic status of patients who respond to chemotherapy may improve spontaneously and a substantial proportion of patients now receive anti-neoplastic therapy even in the last weeks of life. Another aim of the present study was to assess time course changes in regional body fat and lean tissue compartments by analysing computed tomography (CT) images in a subset of pancreatic cancer patients being managed within a regional palliative therapy programme.

3.3 Methods

Patients

All patients referred to the regional cancer centre in Edmonton, Alberta, Canada between January 2004 and October 2008 were considered for the study. Patients with a primary diagnosis of pancreatic cancer entering a palliative programme who had an abdominal CT scan within 60 days of initial assessment were selected for the study (n=111). Patients with ampullary carcinoma, cholangiocarcinoma or neuro-endocrine tumours were excluded. Coding of the primary cancer by its site and morphology, clinical information, and demographic information were obtained from the Alberta Cancer Registry for every patient in the cohort. The Alberta Cancer Registry is a computerised database of all cancer cases in the region (population 1.8 million). Patient-reported height, weight, and weight history were collected during this visit by use of the Patient-Generated Subjective Global Assessment (PG-SGA).(Bauer *et al*, 2002) Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2). Height and weight

recorded by hospital staff on the same date were used for verification where available, and classification of patients' BMI with the use of patient-reporting was found to be accurate. Patient-reported height, weight, and weight history have been shown to be reliable.(Perry *et al*, 1995) Stage of disease was based on the American Joint Committee on Cancer stage groupings I, II, III and IV.

From the initial cohort of 111 patients, 44 patients were further identified who: a) had had at least one further follow-up CT scan, and b) had a documented duration of survival for inclusion into the study of longitudinal changes of body composition.

CT image analysis

CT scans used for analysis were performed solely for routine cancer care. The technique for CT image analysis has been described in detail in chapter 2.

CT dates were expressed in terms of the number of days to death. Any change in tissue area was expressed as either an absolute change (cm^2) or as a percentage change per 100 days. This provided a standardised unit and allowed for comparison across different intervals.

Cut-offs for sarcopenia was based on a CT-based sarcopenic obesity study of cancer patients by Prado *et al.* (2008) (i.e. L3 skeletal muscle index: ≤38.5 cm^2/m^2 for women and ≤52.4 cm^2/m^2 for men).

Statistical analysis

Data are presented as mean ± standard deviation unless otherwise stated. Survival was determined from the time initial assessment until death or until the censor date of 05 January 2009.

Univariate and multivariate survival analysis and calculation of hazard ratios were performed using a Cox regression model. Owing to the large number of covariates examined, only those that were significant on univariate analysis were included in multivariate analysis. Receiver-operator characteristic (ROC) curves were used to select cut-off values for continuous variables. Values with the best combination of sensitivity and specificity were chosen. A backward stepwise procedure was performed to derive a final model of the variables that had a significant relationship with survival. To remove a variable from the model, the corresponding *p*-value had to be greater than 0.05.

Comparisons between groups of patients were assessed using one way ANOVA or Pearson's chi-square test. Survival curves were constructed using the Kaplain-Meier technique. Log-rank test was used to compare survival between groups of patients. Comparison of data at different time points for body composition analysis was performed using the paired t-test. *P* values <0.05 were regarded as statistically significant. Statistical analysis was performed using SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA).

3.4 Results

Details of the 111 pancreatic cancer patients identified at the time of referral to the cancer centre are shown (Table 3.1). About two-thirds of the patients had tumours in the head of

the pancreas. Approximately 75% of patients had biopsy proven adenocarcinoma. At the time of censoring, 101 patients (91.0%) had died. Overall median survival was 130 (interquartile (IQ) range 71 – 302) days. Percentage weight loss in the preceding six months was 12 ± 6% with 89 patients (80.2%) losing more than 5% of their normal body weight. BMI at the time of assessment was 23.9 ± 4.9 kg/m^2 and 44 patients (39.6%) were overweight or frankly obese (BMI ≥25 kg/m^2). Body composition parameters of patients are also reported in Table 1. 62 patients (55.9%) were sarcopenic at this point. 18 patients were overweight/obese (BMI ≥25 kg/m^2) and sarcopenic. The prevalence of sarcopenia within the various BMI categories is presented in Figure 3.1.

Patients were then divided into 4 groups: neither sarcopenic or overweight/obese, overweight/obese, sarcopenic, and both sarcopenic and overweight/obese (Table 3.2). There were no significant differences in age, sex, tumour site, histology, stage of disease, and weight loss between the groups.

Figure 3.1 Venn diagram of BMI classes and sarcopenic patients

Body composition was determined by CT image analysis in patients (n = 111) with advanced pancreatic cancer. Sarcopenia was defined as L3 skeletal muscle index: ≤38.5 cm^2/m^2 for women and ≤52.4 cm^2/m^2 for men.

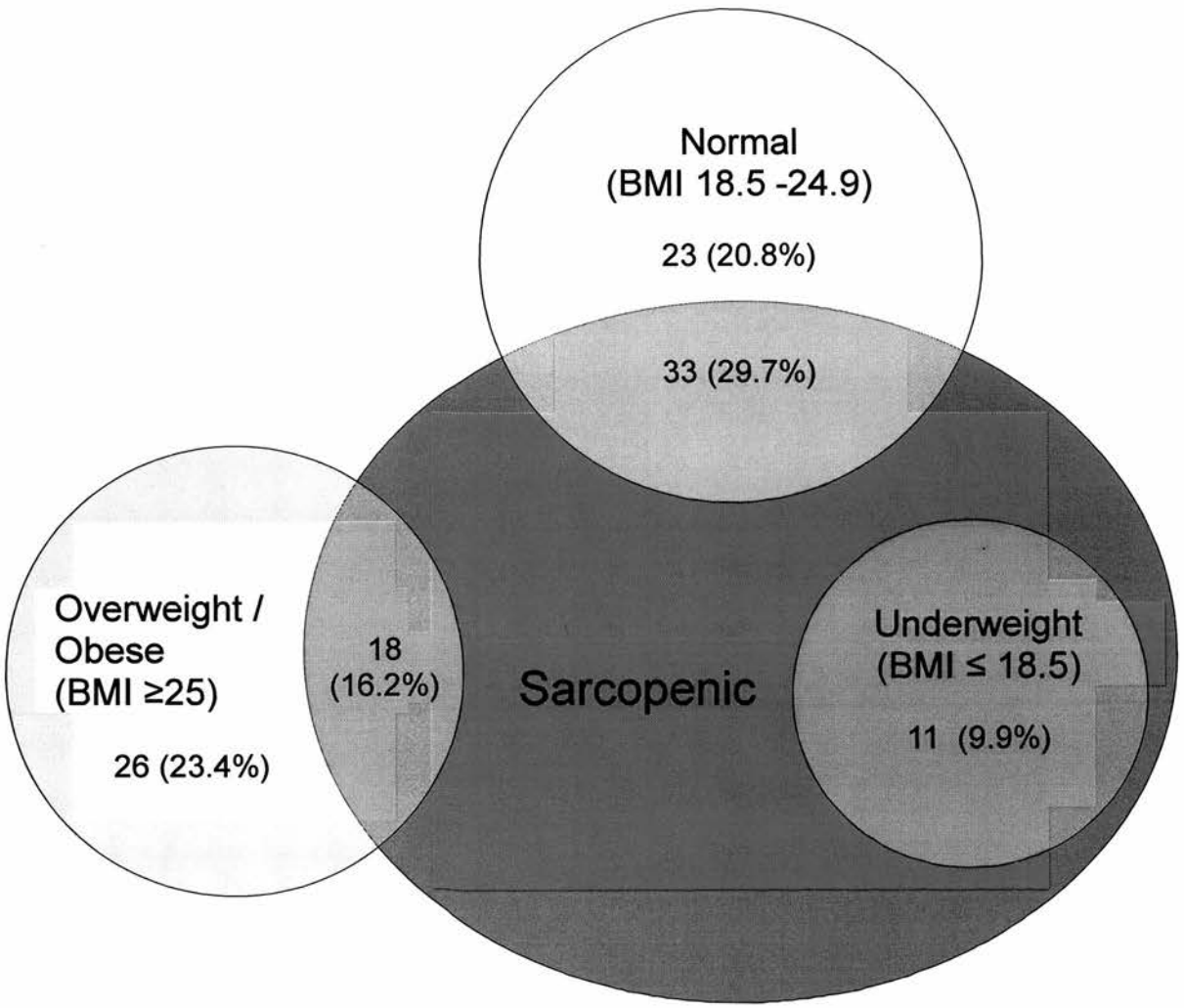


Table 3.1 Overall pancreatic patient (n = 111) demographics, nutritional variables and body composition at the time of entry to palliative care programme

	No. of patients (n=111)
Age (years) †	64.4 ± 9.3
Sex	
M	52 (46.8)
F	59 (53.2)
Tumour site	
Head of pancreas	57 (61.3)*
Body of pancreas	18
Tail of pancreas	4
Pancreatic duct	1
Neck of pancreas	2
Overlapping lesion	11
Not recorded	18
Histology	
Adenocarcinoma	84 (75.7)
Unknown	27
Stage	
II	1
III	7
IV	103 (92.8)
Body mass index (kg/m^2) †	23.9 ± 4.9
Underweight (BMI <18.5 kg/m^2)	11 (9.9)
Normal (BMI 18.5 – 24.9 kg/m^2)	56 (50.5)
Overweight/Obese (BMI ≥25 kg/m^2)	44 (39.6)
Percentage weight loss (in preceding 6 months) †	12.14 ± 6.35
Lumbar total muscle cross sectional area (cm^2) †	126.0 ± 30.7
Lumbar total adipose tissue cross sectional area (cm^2) †	243.7 ± 162.3
Lumbar skeletal muscle index (cm^2/m^2) †	43.8 ± 7.9
Lumbar adipose tissue index (cm^2/m^2) †	86.1 ± 57.4
Estimated total fat-free mass (kg) †	43.9 ± 9.2
Estimated total fat mass (kg) †	21.2 ± 6.9
Sarcopenic	62 (55.9)
Overweight/Obese & sarcopenic	18 (16.2)
Status	
Dead	101 (91.0)
Alive	10

Values are number of patients with percentages in parentheses unless indicated otherwise; †values are mean ± SD; *unknown tumour site was excluded from calculation of overall percentage.

Table 3.2 Comparison of demographic characteristics and body composition of pancreatic cancer patients (n = 111) who were neither overweight nor sarcopenic, sarcopenic alone, overweight alone, and both overweight and sarcopenic

	BMI <25		BMI ≥25		*P*
	Not overweight, not sarcopenic (n = 23; 21%)	Not overweight, sarcopenic (n = 42; 38%)	Overweight/Obese (n = 28; 25%)	Overweight/Obese & sarcopenic (n = 18; 16%)	
Age (years)					
Mean ± SD	60.7 ± 7.5	65.8 ± 10.2	64.3 ± 9.0	66.0 ± 9.3	0.169†
Sex, n (%)					
Male	8 (34.8)	20 (47.6)	11 (39.3)	13 (72.2)	0.084*
Female	15 (65.2)	22 (52.4)	17 (60.7)	5 (27.8)	
Tumour site, n (%)					
Head	8 (34.8)	26 (61.9)	14 (50.0)	9 (50.0)	0.088*
Body	9 (39.1)	3 (7.1)	4 (14.3)	2 (11.1)	
Overlapping lesion	3 (13.0)	3 (7.1)	2 (7.1)	3 (16.7)	
Histology, n (%)					
Adenocarcinoma	18 (78.3)	28 (66.7)	22 (78.6)	16 (88.9)	0.287*
Stage, n (%)					
IV	20 (87.0)	37 (88.1)	28 (100)	18 (100)	0.149*
BMI					
Mean ± SD	22.2 ± 1.9	20.5 ± 2.8	27.6 ± 2.7	28.5 ± 6.3	<0.0001†
% Weight loss					
Mean ± SD	12.83 ± 5.80	13.08 ± 6.45	11.36 ± 6.72	10.45 ± 6.24	0.435†
Lumbar total muscle cross sectional area (cm^2)					
Mean ± SD	129.5 ± 31.8	115.0 ± 29.2	138.5 ± 29.6	128.0 ± 27.6	0.014†
Lumbar total adipose tissue cross sectional area (cm^2)					
Mean ± SD	185.0 ± 137.7	168.8 ± 130.6	344.2 ± 148.0	348.6 ± 155.1	<0.0001†
Lumbar skeletal muscle index (cm^2/m^2)					
Mean ± SD	46.2 ± 7.1	39.3 ± 6.2	49.4 ± 7.3	42.7 ± 6.6	<0.0001†
Lumbar adipose tissue index (cm^2/m^2)					
Mean ± SD	68.4 ± 52.3	57.6 ± 42.0	125.6 ± 55.7	117.1 ± 50.7	<0.0001†
Estimated total fat-free mass (kg)					
Mean ± SD	44.9 ± 9.5	40.6 ± 8.8	47.6 ± 8.9	44.5 ± 8.3	0.014†
Estimated total fat mass (kg)					
Mean ± SD	19.0 ± 5.8	18.3 ± 5.5	25.7 ± 6.2	24.2 ± 7.7	<0.0001†

†one way ANOVA; *Pearson's chi-square test

On univariate analysis, age and overweight/obese sarcopenia were associated with outcome for the patient group. Neither overweight/obese (BMI ≥25 kg/m^2) nor sarcopenia in the non-overweight/obese group failed to reach statistical significance (Table 3.3). In contrast, median survival for patients who were both overweight/obese and sarcopenic was 55 (IQ range 43 – 207) days compared with 148 (IQ range 80 – 369) days for the rest of the patient cohort without overweight/obese sarcopenia (log-rank test, p=0.003) (Figure 3.2). Using ROC curves, cut-off values with the best discriminatory value for age was greater or equal than 59 years.

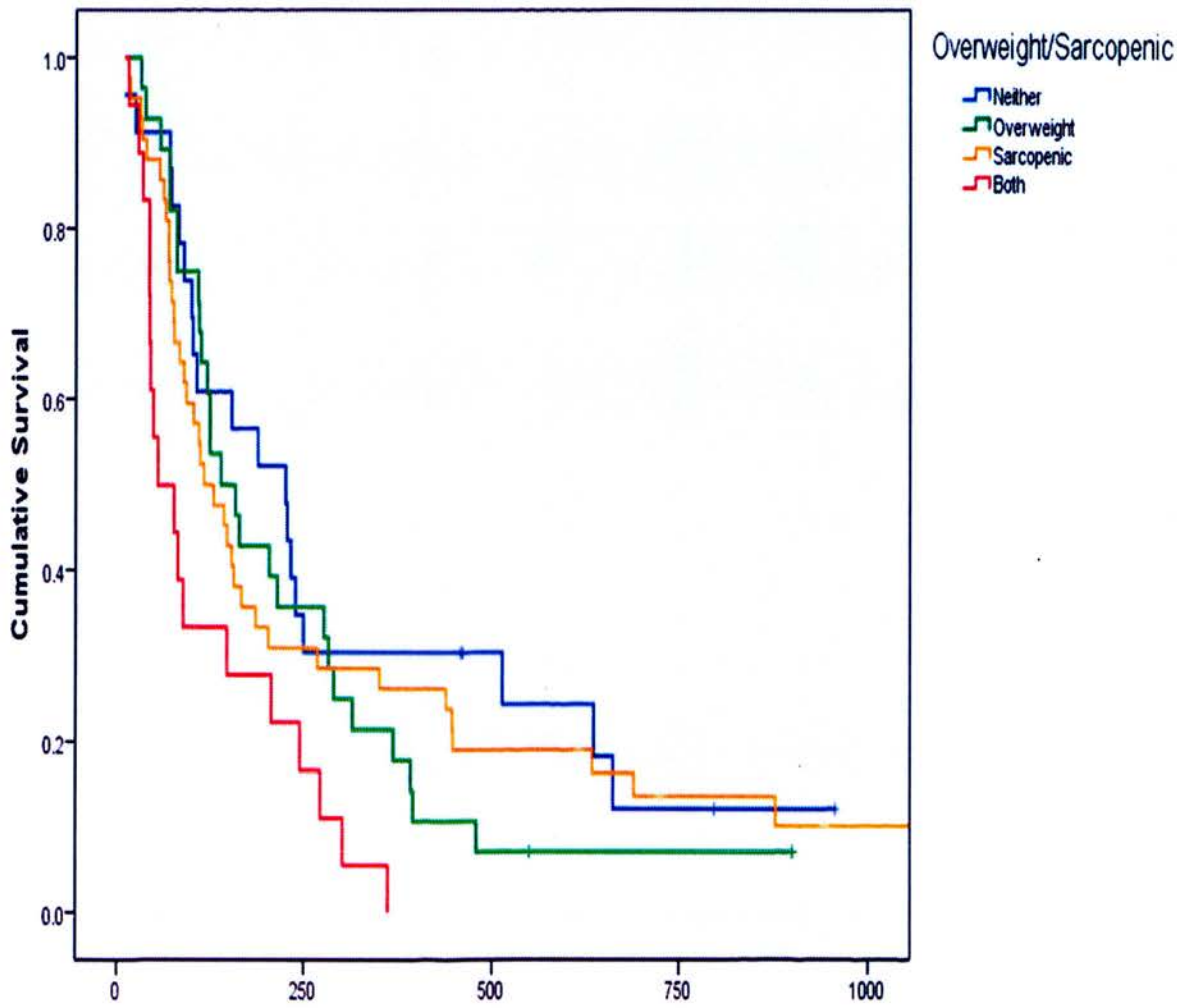
On multivariate analysis, age ≥59 years (HR 1.71, 95% CI 1.10 – 2.66, p=0.018), and overweight/obese sarcopenia (HR 2.07, 95% CI 1.23 – 3.50, p=0.006) retained independent prognostic value (Table 3.3).

Table 3.3 Hazard ratio for risk of death associated with clinical variables and body composition in pancreatic cancer patients (n=111)

	Univariate analysis			*Multivariate analysis*		
	Hazard Ratio	95% C.I	*P**	Hazard Ratio	95% C.I	*P***
Age ≥59 years	1.025	1.002 – 1.048	0.03	1.708	1.097 – 2.660	0.018
Sex	0.793	0.534 – 1.178	0.25			
Tumour site	1.031	0.977 – 1.087	0.268			
Histology	0.791	0.499 – 1.254	0.32			
Stage	1.829	0.910 – 3.677	0.09			
Overweight/obese (BMI ≥25 kg/m^2)	1.454	0.969 – 2.181	0.071			
Percentage weight loss	0.991	0.959 – 1.025	0.599			
Lumbar skeletal muscle index (cm^2/m^2)	1.001	0.976 – 1.026	0.964			
Lumbar adipose tissue index (cm^2/m^2)	1.003	0.999 – 1.007	0.153			
Sarcopenia	1.284	0.863 – 1.910	0.217			
Sarcopenia + overweight/obese	2.177	1.292 – 3.670	0.003	2.071	1.227 – 3.496	0.006

*Cox univariate analysis; **Backward conditional method of Cox proportional hazards model

Figure 3.2 Survival curves of patients who were neither overweight nor sarcopenic, sarcopenic alone, overweight alone, and both overweight and sarcopenic



Number at risk					
Neither	23	7	5	2	0
Overweight	44	12	2	1	0
Sarcopenic	62	17	8	4	2
Both	18	3	0	0	0

Longitudinal analysis of body composition

A subset of 44 patients underwent repeated CT scans as part of their medical management and was therefore available for study of longitudinal changes in body composition. This cohort of patients had a significantly longer survival compared with the entire group (median survival 189 days vs. 130 days, p=0.019 (log-rank test)). 71% of patients with follow-up CT scans received active treatment compared with just 28% of patients who had no follow-up CT scans (p <0.0001, chi-square test). Patients had their 1st CT scan at a mean of 230 ± 62 days before death. The 2nd CT scan was performed at a mean of 95 ± 60 days before death.

The changes in cross sectional area of skeletal muscle and adipose tissue between the two scans are presented in table 4. Patients displayed a significant loss of both skeletal muscle and adipose tissue. Overall, 32 patients (72.7%) lost skeletal muscle and all but one patient (97.6%) lost adipose tissue. The measurements for cross-sectional area were used to estimate whole body fat-free mass (FFM) and whole body fat mass (FM) using regression equations and then normalised for height (kg/m^2) (Table 3.4).

20 patients (45.5%) were sarcopenic at the time of the 1st CT with an estimated FFM index of 15.5 ± 2.5 kg/m^2. By the time of the 2nd CT 27 patients (61.4%) were sarcopenic and FFM had decreased to 14.5 ± 2.0kg/m^2. Estimated FM index decreased from 7.5 ± 2.0 kg/m^2 (1st CT) to 6.0 ± 1.5 kg/m^2 (2nd CT).

Table 3.4 Change in body composition over time in pancreatic cancer patients (n=44).

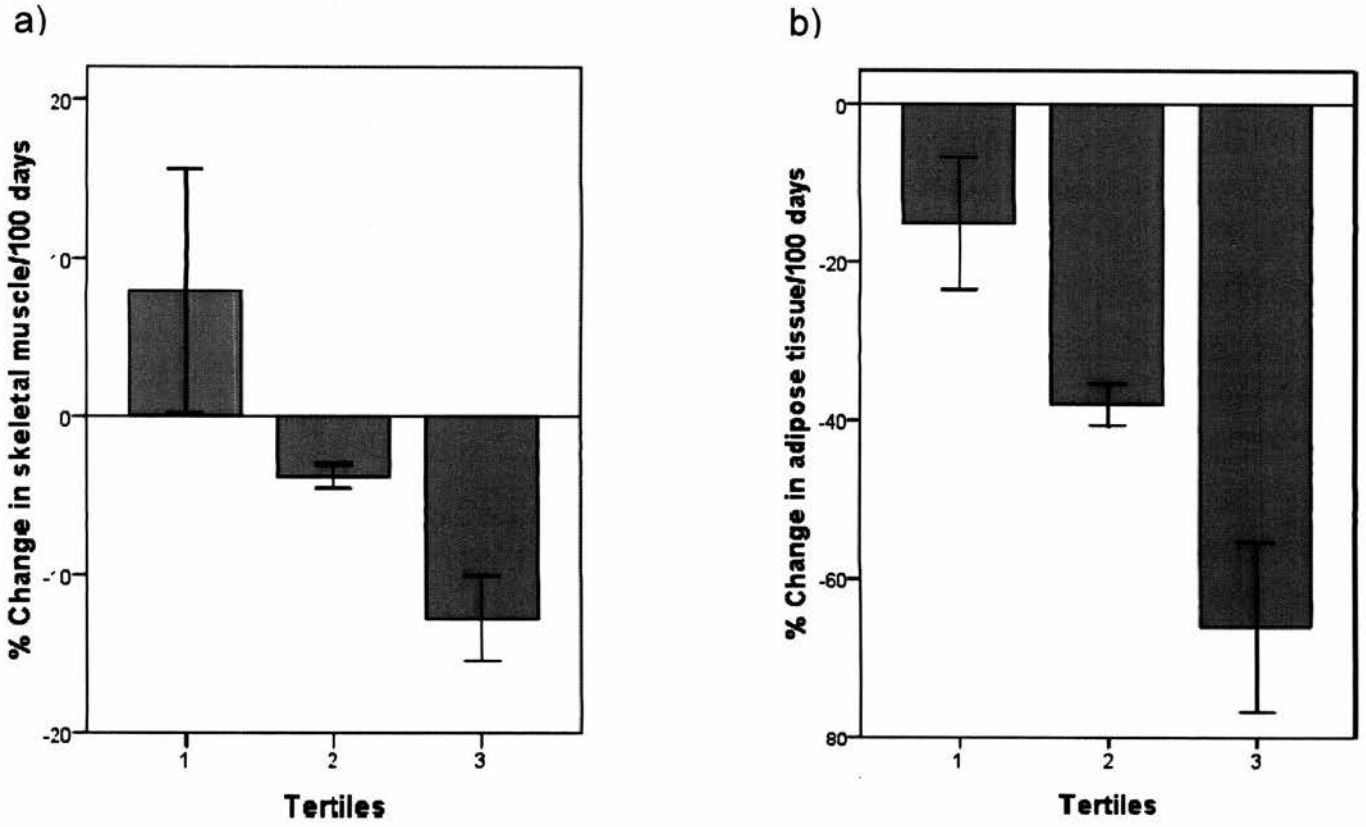
	First CT scan	Second CT scan	Δ	P*
Time to death (days)	230 ± 62	95 ± 60	135 ± 57	
Skeletal muscle (cm^2)	126.5 ± 31.1	119.6 ± 28.6	-7.0 ± 13.6	0.002
Adipose tissue (cm^2)				
Visceral adipose tissue	91.2 ± 69.9	45.4 ± 47.0	-45.8 ± 47.5	<0.0001
Intramuscular and subcutaneous adipose tissue	148.5 ± 85.8	87.0 ± 66.4	-61.5 ± 53.9	<0.0001
Total	236.6 ± 145.5	128.6 ± 102.6	-108.0 ± 89.1	<0.0001
Estimated whole body fat-free mass (FFM) (kg)	44.0 ± 9.3	41.9 ± 8.6	-2.1 ± 4.1	0.002
Estimated whole body adipose tissue (FM) (kg)	21.1 ± 6.1	16.6 ± 4.3	-4.5 ± 3.7	<0.0001
Estimated FFM index (kg/m^2)	15.5 ± 2.5	14.5 ± 2.0	-1.0 ± 1.5	0.002
Estimated FM index (kg/m^2)	7.5 ± 2.0	6.0 ± 1.5	-1.5 ± 1.5	<0.0001

Values are mean ± SD. * Paired t-test

A distinct distribution for muscle and adipose tissue changes over time is more clearly revealed by analysis of population tertiles (Figure 3.3). The overall change of skeletal muscle was -3.1 ± 12.0%/100 days. However, the 1st tertile gained a small amount of muscle tissue (7.9 ± 14.4%/100 days) while the 3rd tertile lost muscle at a rate of -12.7 ± 5.2% /100 days. In comparison, adipose tissue was lost across all 3 tertiles and the overall change of adipose tissue was -40.4 ± 25.4%/100 days (p<0.0001, paired t-test). The proportion of patients receiving chemotherapy was not significantly different across all 3 tertiles of muscle (p=0.372, chi-square test) or adipose tissue loss (p=0.804, chi-square test). Moreover, the changes in muscle (p=0.113, Student's t-test) and fat mass (p=0.862, Student's t-test) were not significantly different between those who did or did not receive chemotherapy. There was also no significant difference in survival across all 3 tertiles for both muscle loss (p=0.142, log-rank test) and adipose tissue loss (p=0.542, log-rank test).

Figure 3.3 Intensity of changes in body composition presented by tertiles

a) Skeletal muscle
b) Adipose tissue



3.5 Discussion

Patients with pancreatic cancer have long been associated with the most severe forms of cachexia. In a similar study undertaken more than 10 years ago, Wigmore *et al.* (1997a) documented median BMI at diagnosis to be 20.7 and this fell to 17.7 near to the time of death. Average weight loss over this time increased from 15% to 25%. Loss of muscle and fat to levels consistent with significant undernutrition increased from 30% to 70% and from 65% to 90% respectively. This was consistent with the conventional view of cancer cachexia (i.e. marked weight loss, severe muscle wasting and gross loss of subcutaneous fat (Fearon & Preston, 1990)). In the present chapter, patients had a mean BMI of 23.9 with 40% of individuals being in the overweight/obese range. Thus, the average physiognomy appears to have changed with patients demonstrating large energy reserves (fat) at the time of presentation with advanced disease. However, underneath this mantle of adipose tissue the previously noted tendency to muscle wasting continues. The use of CT images in the present study identified that 56% of patients had sarcopenia at the time of presentation (Table 3.1) and the tendency to muscle loss continued in at least a proportion of patients (Figure 3.3).

A BMI <18.5kg/m^2 is considered by many authorities to represent an individual at serious risk of undernutrition (Shetty, 2003). In the present chapter, only 10% of individuals at baseline fulfilled this criterion. Given the prevalence of overweight/obesity (40%) it would seem unlikely that even in the presence of ongoing weight loss, the majority would reach this boundary at or near the time of death. However, BMI has clear limitations and more detailed evaluation of body composition clearly revealed wasting of the lean tissues, with a majority of patients below or well below benchmark levels of muscularity known to be associated with mortality and functional disability (Janssen *et al*, 2002). The estimated lean body mass of patients classified as sarcopenic was within the range described for a variety of

wasted/emaciated patient populations with and without malignant disease (Baumgartner *et al*, 1998; Prado *et al*, 2008). In the current literature it is becoming increasingly evident that concurrent sarcopenia and high fat mass is a worst case scenario (Bigaard *et al*, 2004; Heitmann *et al*, 2000; Honda *et al*, 2007; Prado *et al*, 2008), and this is clearly apparent in the study group (albeit small), where sarcopenic overweight/obese patients had the worst prognosis overall, even compared with patients who were sarcopenic and had a lower body weight.

Sarcopenia alone had no discernable effect on mortality yet being overweight/obese and sarcopenic was associated with decreased survival duration. However, a variable can only serve a predictive function where it varies in the relevant population. It should be noted that all patients with a BMI <18.5 were sarcopenic and the majority were sarcopenic in the BMI range 18-5 – 24.9. Thus, one explanation for the finding that sarcopenia was not predictive for the overall population, but was predictive for those with obesity would lie in the differential frequency distribution of sarcopenia in the different BMI categories. Evaluation of this question in a much larger patient population would help resolve this issue.

In the present chapter, patients entering the palliative phase of their pancreatic cancer management had a remarkably high prevalence of sarcopenia compared with reports in the literature for healthy elderly in a similar age bracket (Baumgartner *et al*, 1998); thereby suggesting that substantial muscle wasting had occurred prior to initial evaluation of these patients. The pre-existing nature of this muscle loss makes it difficult to comment as to its mechanisms, except perhaps to suggest that it may be driven by the primary malignancy, by weight loss, and/or by co-morbid conditions which include obesity, insulin resistance, various types of organ failure, and low levels of physical activity. The muscle loss that occurred thereafter, which was characterized during the 135 days scan-scan interval may be driven by disease progression, increased metabolism and inflammation (Falconer *et al*, 1994; Falconer

et al, 1995), and by negative energy balance which might be inferred from the loss of fat mass (i.e. 4.54 kg x ~9000 kcal/kg = 40,840 kcal) in 135 days.

The mechanism that links sarcopenic overweight/obesity with accelerated demise is not known. Muscle wasting is a known complication associated with insulin resistance found commonly in obesity (Wang *et al*, 2006). Adipose tissue synthesises and secretes circulating hormones and 'adipokines' which act as systemic inflammatory mediators and signals of nutritional status (Shoelson *et al*, 2007). These adipocyte factors, such as tumour necrosis factor-alpha (TNFα) and interleukin- 6 (IL-6) are thought to play a major role in the induction of insulin resistance in skeletal muscle leading to an increase in muscle protein loss. The main mediators thought to be involved in this process are inhibitor κB Kinase (IKK) and its downstream effector nuclear factor κB (NF-κB) (Dietze *et al*, 2004). However, not all patients who are overweight/obese have sarcopenia. It may be that cancer-related factors stimulate the initial loss of muscle and being overweight/obese perpetuates and/or enhances muscle loss/loss of muscle function leading to poorer survival. The observation that overweight/obesity may be associated with better survival in patients with weight-losing cardiac failure (Oreopoulos *et al*, 2008) may seem to contradict the present observations in cancer patients. However, the studies in patients with cardiac failure have not been stratified for body composition (specifically sarcopenia) and may represent a disease-specific phenomenon.

Current published results on body composition changes in cancer are varied. Some studies confirm a decline in lean body mass (Maturo *et al*, 2003; Segal *et al*, 2003), while others emphasise a loss of body fat (Fouladiun *et al*, 2005; Lundholm *et al*, 1994). There are also reports that suggest a proportional loss of lean tissue and fat leading to an unchanged body composition (Heymsfield & McManus, 1985; Moley *et al*, 1987). In the present study, roughly half of the patients were found to be already sarcopenic at the time of assessment. Subsequently, a much greater rate of fat loss was noted as compared with muscle loss (see

longitudinal study). In fact, some patients were able to maintain or even gain muscle mass. The patients that gained muscle could have been positive responders to chemotherapy. However, in patients with pancreatic cancer, the use of CT scans to determine response to chemotherapy is complicated by factors such as peritumoural inflammation, therefore duration of survival was regarded as a more robust measure (accepting the small numbers involved in this study). In the present study the survival of patients was not significantly different across all 3 tertiles of loss of muscle mass, thus response to chemotherapy as the main reason for patients to gain muscle mass cannot be confirmed. An alternative hypothesis would be that a proportion of patients were able to activate compensatory mechanisms aimed at conserving muscle. There is increasing evidence that gene polymorphisms are related to cancer cachexia susceptibility (Deans *et al*, 2009a; Vigano *et al*, 2009).

Systemic inflammation is known to be a key mediator in cachexia (Stephens *et al*, 2008), and has been associated with poor prognosis in previous studies on pancreatic cancer (Fearon *et al*, 2006; Glen *et al*, 2006). A limitation of the present study is the lack of measures of systemic inflammation such as C-reactive protein (CRP) which may be significant when assessing factors influencing survival in pancreatic cancer.

Nevertheless, this study has shown that sarcopenia in overweight/obese advanced pancreatic cancer patients may be an occult condition but can be identified using diagnostic CT scans and is an independent adverse prognostic indicator. Longitudinal analysis of body composition in pancreatic cancer patients revealed a loss of both skeletal muscle and adipose tissue in the course of the cancer journey but, adipose tissue was lost more rapidly than muscle.

CHAPTER 4

IDENTIFICATION OF POSSIBLE GENETIC VARIANTS INVOLVED IN CANCER CACHEXIA: A SYSTEMATIC LITERATURE REVIEW

4.1 Summary

Aims

The aims of this chapter are to explore genetic polymorphisms with known functional or clinical significance in potential candidate genes involved in the development of cancer cachexia by means of a systematic literature review, and to identify the polymorphisms with the most likely potential as susceptibility markers for cancer cachexia.

Methods

A systematic search of the Medline and EmBase databases, covering 1986 – 2008 was performed for potential candidate genes/genetic polymorphisms relating to cancer cachexia. Related genes were then identified using pathway functional analysis software. All candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms associated with cachexia using the OMIM and GeneRIF databases.

Results

A total of 184 polymorphisms with functional or clinical relevance to cancer cachexia were identified in 92 candidate genes. Of these, 42 polymorphisms (in 33 genes) were replicated in more than one study with 13 polymorphisms found to influence two or more hallmarks of cachexia (i.e. inflammation, loss of fat mass and/or lean mass, reduced survival).

Conclusions

Selection of candidate genes and polymorphisms is a key element of multigene study design. The present chapter provides an initial framework in which to select genes/polymorphisms for further study in cancer cachexia (see chapter 6).

4.2 Introduction

Certain tumour types are more commonly associated with cachexia, but even with the same tumour type there are variations in the extent to which patients exhibit cachexia. Such variation may, in part, be due to the patient's genotype rather. It is therefore likely there may be a cachexia prone genotype as well as a cachexia protective genotype.

The cachexia syndrome is thought to result from a complex interplay of mechanisms involving the initiation of a host inflammatory response mediated by tumour-derived proinflammatory cytokines; the reprioritisation of protein metabolism with induction of the acute phase response and mobilisation of fat reserves; and activation of neuroendocrine pathways which may lead to hypermetabolism and increased catabolism (Tan *et al*, 2008). The wealth of known genetic variation in genes regulating the above mechanisms suggests their exploitable potential as biomarkers of inter-individual predictability of developing cachexia.

Single nucleotide polymorphisms (SNPs) are the most common type of stable genetic variation in the population (Brookes, 1999). SNPs occur in approximately one in every

thousand bases. There are several ways that SNPs can lead to an aberrant gene product. Promoter polymorphisms that alter DNA binding of transcription factors have the potential of decreasing or increasing gene expression; Sequence variation in the 5` untranslated region (UTR) could disrupt mRNA translation, and mutations in the 3` UTR could affect mRNA cleavage, stability, and export; Finally, non-synonymous SNPs in exons could alter protein function or activity. It has been estimated that 10% of all SNPs in the genome are functional, thereby having the potential of altering some biological process (Wjst, 2004).

Over the past 10 years, considerable information has emerged on mechanisms of cancer cachexia of which inflammation is postulated to play a significant role. These mechanisms can be broadly grouped into five domains of interest: systemic inflammation, central energy balance, control of muscle metabolism/function, control of adipose tissue metabolism/function, and regulation of appetite.

This chapter aims to explore genetic polymorphisms with known functional or clinical significance in potential candidate genes involved in the development of cancer cachexia within the above-mentioned domains by means of a systematic literature review. It also aims to identify the polymorphisms with the most likely potential as susceptibility markers for cancer cachexia.

4.3 Methods

The scientific literature published between 1986 and 2008 was searched in Medline and EmBase databases. In order to maximise the potential of identifying potential candidate genes, search terms were utilised that took into the account the effects of cachexia. E.g. 'survival' as a surrogate for accelerated death which may be due to cachexia. The term 'body composition' was used to identify potential genes of interest that may predispose individuals to higher/lower body mass which may influence the propensity to the development of cancer cachexia. 'Inflammation' was used as a main search term due to its postulated central role in the development of cancer cachexia.

Overall, the following search terms were used to identify potential candidate genes/polymorphisms of interest:

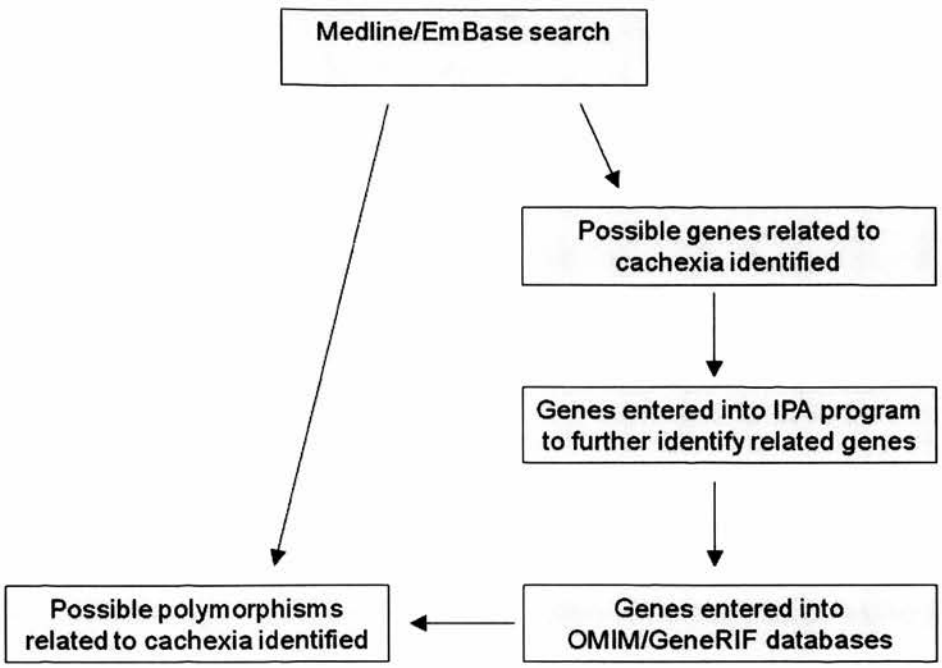
- ((genes/genetics) OR polymorphism(s)) AND (inflammation OR cancer OR cachexia OR weight loss OR body composition OR survival)

The search was limited to English language and humans.

Following the initial retrieval of possible candidate genes, the genes were entered into a pathway functional analysis software (Ingenuity Pathways Analysis (IPA), Ingenuity Systems, California) to further identify related genes.

All identified candidate genes were then reviewed for functional polymorphisms or clinically significant polymorphisms in terms of cachexia using OMIM and GeneRIF databases.

Figure 4.1 Search strategy employed to identify genetic variants associated with cachexia



Candidate gene variants were grouped into categories according to genes that regulate or code for the following:

- Inflammation
    - Innate immune receptors and mediators of the immune response
    - Cytokines
    - Cytokine receptors and related binding proteins
    - Acute phase protein reactants
- Central homeostasis
    - Energy production
    - Insulin like growth factors and related proteins
    - Corticosteroid signalling proteins

- Muscle
    - Muscle function and structure
    - Muscle proteolysis
- Adipose tissue
    - Adipogenesis
    - Lipid turnover and transport
    - Adipokines and adipokine receptors
- Appetite
- Others

Summary tables of polymorphisms are also presented according to each category with 'easy to see' boxes that denote whether a polymorphism has any effect on inflammation, weight/body composition (i.e. lean mass/fat mass) and cancer survival. The summary tables also denoted if a functional or clinical association with a polymorphism was replicated in more than one study. In addition polymorphism reference numbers (rs numbers) were also recorded if known, as well as the minor allele frequency of the polymorphism based on a population with European ancestry derived from the HapMap or dbSNP databases

4.4 Results

A total of 184 polymorphisms with functional and/or clinical significance in terms of cachexia were identified in 92 genes.

Inflammation

Inflammation has long been associated with cancer cachexia. Systemic inflammation could result from tumour cell or host cell mediated production (Stewart *et al*, 2006). In experimental models, pro-inflammatory cytokines such as interleukin 1-beta (IL-1β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNFα), and interferon gamma (IFNγ) may lead to an acute phase response and trigger tissue catabolism (Argiles *et al*, 2003).

Innate immune receptors and mediators of the immune response

Table 4.1 explores the possible genetic determinants in the generation or suppression of the inflammatory response and how they may relate to cancer cachexia. These include variants in genes coding for the Toll-like receptor (TLR) family, and associated genes, which play an instructive role in innate immune responses as well as the subsequent induction of adaptive immune responses. TLRs are involved in triggering intracellular signals, culminating in the activation of nuclear factor (NF)-κB, where it participates in enhancing expression of other immuneregulatory substances (Kawai & Akira, 2006). NF-κB is a transcriptional regulator that plays a central part in responses to inflammatory signalling. Polymorphisms in genes encoding for NF-κB and genes involved in the activation or inhibition of NF-κB are also shown in table 4.1.

Also of interest are variants in genes coding for cell adhesion molecules (CAMs) which are proteins located on the cell surface involved with the binding with other cells or with the extracellular matrix. CAMs are known to mediate migration of cells to sites of inflammation. Functional polymorphisms in the heat shock proteins genes HSPA1L and HSPA1B have been noted in relation to inflammation and these are also displayed in table 4.1.

Table 4.1 Summary of polymorphisms involving innate immune receptors and mediators of the immune response

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TLR1	• T1805G (602 Ile→Ser) **[rs5743618]**	16%	• Impaired basal and lipopeptide induced NFκB signalling (Hawn *et al*, 2007)	↓				
TLR2	• C2029T(677 Arg→Trp)	?	• Decreased production of IL-12 when stimulated with *M.Leprae* (Kang & Chae, 2001) • Inhibition of NFκB activation (Bochud *et al*, 2003)	↓				✓
	• G2251A (753 Arg→Gln) **[rs5743708]**	5%	• Decreased NFκB activation (Lorenz *et al*, 2000)	↓				
	• A-16934T **[rs4696480]**	<1%	• Increased GT repeats in intron1 of TLR2 (Veltkamp *et al*, 2007) • Decreased PMBC production of TNF-α, IL-6, IL-12 (Veltkamp *et al*, 2007)	↓				
TLR4	• A896G (299 Asp→Gly) **[rs4986790]**	4%	• Significantly reduced levels of NFκB translocation (Arbour *et al*, 2000) • Reduced levels of pro-inflammatory cytokines, acute phase reactants (Kiechl *et al*, 2002)	↓				✓
TLR5	• C1174T (392 Arg→TER) **[rs5744168]**	8%	• Decreased NFκB activation in response to flagellin (Hawn *et al*, 2003)	↓				
LY96	• A103G (35 Thr→Ala)	?	• Decreased TNF-α levels after *in vitro* stimulation with LPS (Hamann *et al*, 2004)	↓				
	• C-1625G	?	• Increased expression of MD-2 mRNA and production of TNFα in whole blood leukocytes, in response to LPS stimulation (Gu *et al*, 2007)	↑				
CD14	• C-159T **[rs2569190]**	47%	• Increased levels of sCD14 (Baldini *et al*, 1999) • Lower levels of IgE (Baldini *et al*, 1999) • Higher PMBC TNF-α mRNA levels after incubation with LPS/*E.coli* (Temple *et al*, 2003)	↑				

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IRAK4	• C877T (287 TER) • 620–621del (293 TER)	?	• Impaired activation of NFκB and cytokine production (Medvedev *et al*, 2003)	↓				
MBL2	• G-289C (Y/X) **[rs7096206]**	22%	• Low serum MBL levels (Garred *et al*, 2003)				↑	
	• 52 Arg→Cys (D allele) **[rs5030737]** • 54 Gly→Asp (B allele) **[rs1800450]** • 57 Gly→Glu (C allele) **[rs1800451]**	7% 15% 2%	• Low serum MBL levels (Madsen *et al*, 1995; Super *et al*, 1989)	↑				✓
ICAM1	• A1548G (469 Lys→Glu) **[rs5498]**	40%	• G/G cells expressed lower amount of ICAM-1-mRNA than A/A cells (Iwao *et al*, 2004) • Lower fibrinogen levels (Yokoyama *et al*, 2005)				↑	
VCAM1	• A-540G **[rs3783605]**	<1%	• ETS2 transcription factors were found to be bound more significantly to G allele (Idelman *et al*, 2007)				↓	
SELP	• A37674C (715 Thr→Pro) **[rs6136]**	9%	• Decreased serum P-selectin levels (Miller *et al*, 2004; Volcik *et al*, 2006)	↓			↑	✓
	• C-2123G • A-1969G	? ?	• Decreased serum P-selectin levels with -2123C and -1969G (Barbaux *et al*, 2001)	↓			↑	
HSPA1L	• C2347T (493 Thr →Met) **[rs2227956]**	24%	• Increased plasma IL-6 levels and TNF-α levels with C allele (Schroder *et al*, 2003)	↓				
HSPA1B	• C-179T	?	• Elevated mRNA levels of HSPA1A and HSPA1B (Temple *et al*, 2004)					
	• A1538G	?	• Increased plasma IL-6 levels and TNF-α levels with A allele (Schroder *et al*, 2003)	↓				

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
NFKB1	• -94 ins/del **[rs28362491]**	?	• I allele promoter had significantly higher activity with reporter gene assays (Karban *et al*, 2004; Park *et al*, 2007)	↓				✓
NFKBIA	• A/G (3' UTR) **[rs696]**	39%	-				↓	
AKT1	• G205T (5' UTR) **[rs1130214]**	28%	• Greater AKT1 gene expression (Ludlow *et al*, 2007)			↑		
AGER	• 82 Gly→Ser **[rs2070600]**	6%	• Higher levels of plasma TNF-α, serum CRP with Ser/Ser genotype (Jang *et al*, 2007b)	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Cytokines and cytokine receptors

Cytokines are secreted proteins that play a role in the induction and effector phases of all immune and inflammatory responses. They serve diverse functions including induction of cell proliferation, mediating intercellular communication, chemotaxis and cell killing. Genetic variants of genes encoding pro- and anti-inflammatory cytokines are presented in Table 4.2.

Table 4.2 Summary of cytokine polymorphisms

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TNF	• G-308A **[rs1800629]**	17%	• Increased TNF-α production (Sallakci *et al*, 2005) • Six fold increase in transcription of TNF-α (Wilson *et al*, 1997)	↑	↑			✓
	• G-238A **[rs361525]**	7%	• Decreased transcriptional activity (Kaluza *et al*, 2000) • Decreased PMBC production of TNF-α after stimulation with T-cell mitogens (Kaluza *et al*, 2000) • Decreased insulin resistance (Day *et al*, 1998)	↓	↓			
	• C-857T **[rs1799724]**	4%	• Increased serum TNF-α levels (Gonzalez-Quintela *et al*, 2004)	↓	↓			
	• C-863A **[rs1800630]**	15%	• Reduced total serum IgE levels (Sharma *et al*, 2006) • Reduced serum TNF-α levels (Sharma *et al*, 2006) • 31 % decrease in transcription of TNF-α (Skoog *et al*, 1999)	↓	↓			✓
LTA	• A252G (Intron 1) **[rs909253]**	36%	• Increased serum TNF-α levels (McArthur *et al*, 2002; Stuber *et al*, 1996)	↑			↓	✓
IL1A	• G4845T (114 Ala→Ser) **[rs17561]**	26%	• Pre IL-1α with Ala more resistant to proteases in human sera compared with Pre IL-1α with Ser (Kawaguchi *et al*, 2007)					
	• C-889T **[rs1800587]**	25%	• Significantly increased transcriptional activity of the IL-1α gene with TT genotype (Dominici *et al*, 2002) • Slight increase of IL-1α mRNA and protein levels (Dominici *et al*, 2002)	↑				

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IL1B	• C-31T **[rs1143627]**	36%	• Increased expression of IL-1β gene with T allele (Lind *et al*, 2007) • Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al*, 2006)	↑			↑	✓
	• C-511T **[rs16944]**	36%	• Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • No significant increase in IL-1β production in response to LPS in patients homozygous for T allele (Awomoyi *et al*, 2005)	↓			↓	✓
	• G-1470C	?	• Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al*, 2006)	↓				
	• C3953T (3954) (synonymous) **[rs1143634]**	21%	• T/T genotype associated with lower plasma levels of IL1-RA (Tolusso *et al*, 2006) • Increased human amniochorion IL-1β production after stimulation with LPS (Hernandez-Guerrero *et al*, 2003)	↑	↓		↓	✓

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IL4	• C-33T **[rs2070874]**	14%	• Increase in serum IL-4 levels (Gervaziev *et al*, 2006)	↑				
	• C-590T **[rs2243250]**	14%	• Higher gene reporter assay activity (Rosenwasser *et al*, 1995) • Different pattern of protein binding on electrophoretic mobility shift assay (Rosenwasser *et al*, 1995) • Higher serum levels of IgE (Rosenwasser *et al*, 1995)	↑				
IL6	• G-174C **[rs1800795]**	46%	• Lower levels of IL-6 in plasma in healthy subjects (Fishman *et al*, 1998) • Higher fasting plasma insulin levels with G allele (Yang *et al*, 2005) • Lower circulating adiponectin levels with G allele (Yang *et al*, 2005)	↓	↑	↓		✓
	• 32 Pro→Ser **[rs2069830]** • 162 Asp→Val **[rs2069860]**	<1% 2%	• Lower levels of IL-6 (Liu *et al*, 2006) • Higher levels of albumin (Liu *et al*, 2006)	↓				
IL8	• A-251T **[rs4073]**	40%	• Increased IL-8 production in whole blood stimulated with LPS with A allele (Hull *et al*, 2000)	↓	↓			
IL10	• C-592A **[rs1800872]**	21%	• Significantly reduced levels of IL-10 (Lowe *et al*, 2003)	↑				
	• G-1082A **[rs1800896]**	47%	• Increased levels of IL-10 with -1082GG (Schaaf *et al*, 2003) • -1082GG associated with increased CRP (Deans *et al*, 2007a)	↕			↑	
IL12B	• A16974C (3' UTR) **[rs3213113]**	<1%	• Higher IL-12 secretion levels from LPS and PPD stimulated PBMCs in C/C genotype (Yilmaz *et al*, 2005)	↑				

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IL13	• C-1055T **[rs1800925]**	19%	• Decreased inhibition of IL-13 production upon stimulation with anti-CD2 (van der Pouw Kraan *et al*, 1999) • Increased binding of nuclear proteins (van der Pouw Kraan *et al*, 1999)	↓				
IL18	• A105C (synonymous) **[rs549908]** • G-137C **[rs187238]**	33% 30%	• Increased IL-18 production from LPS and A23187 + PMA stimulated monocytes in 105AA and -137GG (Arimitsu *et al*, 2006)	↓				
	• C/T **[rs5744256]**	21%	• Reduced serum concentrations of IL-18 (Frayling *et al*, 2007)	↓				
	• G-9731T **[rs1946519]** • T-5848C **[rs2043055]** • A4860C **[rs549908]** • T8855A **[rs360729]** • A11015C **[rs3882891]**	39% 41% 33% 33% 41%	• Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (Thompson *et al*, 2007)	↑	↓			
IFNA2	• -300 to -305 del • -347 to -343 del	? ?	• Lower level of transcription with reporter gene assays (Song le *et al*, 2006)	↓				
IFNG	• T874A (Intron1) **[rs2430561]**	?	• Decreased mRNA levels of IFN-γ and IL-6 (Biolo *et al*, 2006) • Lower frequency of CRP elevation (Biolo *et al*, 2006) • Preferential binding of NFκB to T allele (Rossouw *et al*, 2003)	↓				✓
	• Intron 1 CA repeat polymorphism 12 CA repeats (Allele 2)	?	• Increased production of IFN-γ *in vitro (Pravica et al, 1999)*	↑				
	• G-179T **[rs2069709]**	<1%	• Increased reporter gene assay activity in response to TNF-α (Bream *et al*, 2002)	↑				

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TGFB1	• T29C (10 Leu→Pro) **[rs1982073]**	<1%	• Significantly higher fasting insulin values as well as higher insulin-resistance in heterozygotes (Rosmond *et al*, 2003)		↑			
	• C-509T **[rs1800469]**	29%	• -509C exclusively binds AP1 which downregulates expression of TGF-β1 promoter (Shah *et al*, 2006)	↑			↑	
TGFB2	• -246ins	?	• Increased transcriptional activity of TGF-β2 promoter with reporter gene assays (Beisner *et al*, 2006) • -246ins binds Sp1 which increases transcription activity (Beisner *et al*, 2006)	↑			↓	
GDF15	• C2423G (6His→Asp) **[rs1058587]**	1%	-				↓	
MIF	• G-173C **[rs755622]**	15%	• Higher serum and synovial fluid levels of MIF (De Benedetti *et al*, 2003)	↑			↕	
CCL2	• A-2518G (A-2578G) **[rs1024611]**	30%	• Increased transcription of CCL2 gene with reporter gene assay (Rovin *et al*, 1999) • Associated with higher serum MCP-1 level (McDermott *et al*, 2005)	↑				✓
CCL5	• G-403A **[rs2107538]**	16%	• G allele associated with higher serum levels of RANTES (Jang *et al*, 2007a) * Serum RANTES correlates with CRP levels (Jang *et al*, 2007a)	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Cytokines exert their effects via matching cell-surface receptors. Subsequent cascades of intracellular signalling then alter cell functions. This may include the upregulation and/or downregulation of several genes and their transcription factors, resulting in the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition (Ihle, 1995). Table 4.3 summarizes the variations in genes encoding cytokine receptors and related binding proteins.

Table 4.3 Summary of polymorphisms of cytokine receptors and related binding proteins

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TNFRSF1A	• G-329T **[rs4149570]**	34%	• T allele results in repression of TNFSFR1A expression (Kim *et al*, 2008)	↓	↓			
	• A36G (synonymous) **[rs767455]**	49%	• Increased TNFR1 levels with 36GG (Mavri *et al*, 2007)	↑	↑			
TNFRSF1B	• 196 Met→Arg **[rs1061622]**	24%	• Significantly lower capability to induce TNFR2-mediated NFκB activation (Till *et al*, 2005)	↓				
	• G1663A **[rs1061624]** • T1668G **[rs5030792]**	48% ?	• Increased gene transcription with 1663G, 1668T, 1690C haplotype (Puga *et al*, 2005)	↓	↓			
	• T1690C **[rs3397]**	47%	• Increased gene transcription with 1663G, 1668T, 1690C haplotype (Puga *et al*, 2005)	↑	↑			
IL1R1	• A1622G	?	• Reduced plasma levels of IL1-R1 (Bergholdt *et al*, 2000)	↓				
IL1RN	• Variable 86bp repeat polymorphism Intron 2 Allele 2 (240bp)	-	• Increased levels of IL-1RA in monocytes (Danis *et al*, 1995) • Increased serum leptin (Strandberg *et al*, 2006)	↓	↑			
IFNGR1	• T-56C **[rs2234711]**	35%	• Lower transcription of IFNGR1 promoter with reporter gene assays (Juliger *et al*, 2003)	↓				
LTBP1	• G-202C • A20C	? ?	• Increased transcriptional activity with -202G and 20A (Higashi *et al*, 2006)	↓				
LITAF	• 174Ala→Ser	?	• Elevated LITAF mRNA expression in extramammary Paget's disease tumour sample (Matsumura *et al*, 2004) * Inhibition of LITAF mRNA expression in THP-1 cells resulted in a reduction of TNF-α transcripts (Myokai *et al*, 1999)	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Acute phase protein reactants

Acute-phase protein reactants (APPR) are proteins whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation (Stephens *et al*, 2008), and are also predictors of adverse outcomes in cancer patients. Some APPR also have roles in modulating the immune response such as C-reactive protein (CRP). Variants in genes coding for APPR are shown in table 4.4.

Table 4.4 Summary of polymorphisms of acute phase proteins

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
CRP	• G1059C (synonymous) **[rs1800947]**	7%	• Lower plasma CRP concentration with C/C genotype (Dai *et al*, 2007; Thalmaier *et al*, 2006)				*	✓
	• A1009G (-717) **[rs2794521]**	34%	• Lower serum concentration on CRP (Sheu *et al*, 2007)					
	• Polymorphic GT repeats (Intron 1)	?	• GT^{16} & GT^{21} alleles associated with lower serum CRP (Szalai *et al*, 2002)					
	• C1444T (3'UTR) **[rs1130864]**	30%	• Higher CRP concentrations (Brull *et al*, 2003; D'Aiuto *et al*, 2005)					✓
ALB	• C4446T (114 Arg→TER)	?	• Analbuminaemia (Campagnoli *et al*, 2005)				*†	
	• 2430-31 AT del	?	• Analbuminaemia (Campagnoli *et al*, 2006)					
	• G118A	?	• Analbuminaemia (Campagnoli *et al*, 2002)					
FGB	• G-455A **[rs1800790]**	26%	• Increased plasma fibrinogen levels (Tybjaerg-Hansen *et al*, 1997)		↑			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

* CRP is a predictive indicator of survival time of terminally ill cancer patients (Suh & Ahn, 2007)

*† Glasgow Prognostic Score (GPS) links low albumin and high CRP to survival in patients with inoperable gastro-oesophageal cancer (Crumley *et al*, 2006b); GPS is a significant predictor of survival in inoperable pancreatic cancer (Glen *et al*, 2006)

Central homeostasis

Body mass is controlled by the balance of energy intake and expenditure, like all thermodynamic systems. In certain forms of cancer, patients with cachexia have been observed to have much higher resting energy expenditure (REE) (Fredrix *et al*, 1991). Gene polymorphisms in the regulatory pathways controlling energy intake and expenditure are discussed below. The following section also explores genes involved in growth and development, and metabolic pathways common to both muscle and adipose tissue.

Energy production

Uncoupling proteins (UCPs) are transporters, present in the mitochondrial inner membrane, that mediate a regulated discharge of the proton gradient that is generated by the respiratory chain. This energy-dissipatory mechanism can serve functions such as thermogenesis, maintenance of the redox balance, or reduction in the production of reactive oxygen species (Ledesma *et al*, 2002). There are a total of five UCP homologs in humans. There are known significant polymorphisms within three UCP genes that may have a role in the development of cachexia and these are presented in table 4.5.

A polymorphism in the gene coding for Triose phosphate isomerase (TPI) which plays an important role in glycolysis and is essential for efficient energy production is also shown in table 4.5. TPI deficiency leads to a metabolic block of the glycolytic pathway and hence a generalized impairment of cellular energy supply causing generalised skeletal muscle deficiency.

Table 4.5 Summary of polymorphisms regulating energy production

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
UCP1	• A-112C **[rs10011540]**	9%	• Increased insulin resistance (Fukuyama *et al*, 2006)					
	• A-3826G **[rs1800592]**	25%	• Reduced mRNA expression of UCP1 (Sramkova *et al*, 2007)		↑			
UCP2	• G-866A **[rs659366]**	37%	• Increased UCP2 mRNA expression and transcriptional activity (Esterbauer *et al*, 2001; Krempler *et al*, 2002) • Increased insulin resistance with A/A genotype (D'Adamo *et al*, 2004)		↓			✓
	• C164T (55 Ala→Val) **[rs660339]**	42%	• Increased plasma leptin (Rance *et al*, 2007) • Increased fasting insulin with Val/Val genotype (Wang *et al*, 2007)		↑			
	• 45bp ins/del polymorphism (exon 8)	?	• Significant leptin resistance with del/del genotype (Yanagisawa *et al*, 2006)		↓			✓
UCP3	• C-55T **[rs1800849]**	24%	• Higher skeletal muscle UCP3 mRNA expression (Schrauwen *et al*, 1999)		↓			✓
TPI1	• G/C (104 Glu→ Asp) **[rs28934569]**	?	• Associated with TPI deficiency (Arya *et al*, 1997; Daar *et al*, 1986)			↓		✓

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Insulin like growth factors and related proteins

The insulin-like growth factors (IGFs) are polypeptides with high sequence similarity to insulin. IGFs are part of a complex system that cells use to communicate with their physiologic environment. This complex system (often referred to as the IGF "axis") consists of two ligands (IGF-1 and IGF-2), two cell-surface receptors (IGF1R and IGF2R), and a family of six high-affinity IGF binding proteins (IGFBP 1-6) (Jones & Clemmons, 1995). This system regulates normal cellular metabolism, proliferation, differentiation and protecting against apoptotic signals (Jerome *et al*, 2003). High levels of IGF-1 and IGFBP3 have been implicated with poorer prognosis in certain types of cancers. Polymorphisms in genes coding for components of the IGF axis have been shown to affect serum levels of their respective proteins as well as body composition (Table 4.6).

Also shown in table 4.6 are polymorphisms in the iodothyronine deiodinase, type 1 gene (DIO1) which activates thyroid hormone by converting the prohormone thyroxine (T4) by outer ring deiodination to bioactive triiodothyronine (T3). Thyroid hormone is known to interact with the GH-IGF-1 axis although the exact mechanism is unknown (Peeters *et al*, 2005).

Table 4.6 Summary of polymorphisms of insulin like growth factors and related proteins

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IGF1	• CA repeat promoter polymorphism 19 CA repeats (192bp)	?	• Lower serum IGF-I levels (Morimoto *et al*, 2005; Rosen *et al*, 1998) • Lower IGFBP-3 levels (Morimoto *et al*, 2005)		↓		↑	✓
IGF2	• G820A (G17200A) (3'UTR) **[rs680]**	2%	• Higher serum IGF-II concentration in A/A genotype (O'Dell *et al*, 1997)		↓			
IGF2R	• ACAA-insertion/deletion (3' UTR) **[rs8191962]** Wt – 144bp Mutant – 140bp	?	• Lower insulin and triglyceride levels with homozygous Wt (Villuendas *et al*, 2006)					
IGFBP3	• C2133G (32 Ala→Gly) **[rs2854746]**	38%	• Increased IGFBP3 levels (Le Marchand *et al*, 2005; Morimoto *et al*, 2005)				↓	✓
	• C-202A **[rs2854744]**	41%	• Increased IGFBP3 levels (Le Marchand *et al*, 2005; Schernhammer *et al*, 2003)				↓	✓
IRS1	• 971 Gly→Arg **[rs1801278]**	6%	• Decrease in IRS-1 associated PI3-kinase activity (Almind *et al*, 1996) • Decrease in binding of the p85 regulatory subunit of PI3-kinase to IRS-1 (Almind *et al*, 1996) • Decrease in incorporation of thymidine into DNA (Almind *et al*, 1996)		↓			
DIO1	• C785T (3'UTR) **[rs11206244]**	36%	• Lower activity of D1, higher levels of free IGF-I with haplotype 785T, 1814A (Peeters *et al*, 2005)			↑		
	• A1814G (3'UTR) **[rs12095080]**	10%	• Lower activity of D1, higher levels of free IGF-I with haplotype 785T, 1814A (Peeters *et al*, 2005)					

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Corticosteroid signalling proteins

Corticosteroids are essential steroid hormones that are secreted by the adrenal cortex and affect multiple organ systems. Corticosteroids are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation, carbohydrate metabolism and protein catabolism. The genetic variants of the components in the mechanism of corticosteroid signalling are examined in table 4.7.

Table 4.7 Summary of polymorphisms of corticosteroid signalling proteins

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
GCCR	• A3669G	?	• Increased stability of GRβ mRNA and increased GRβ protein expression (Syed *et al*, 2006)		↓			
	• A1220G (363 Asn→Ser) **[rs6195]**	3%	• Associated with increased glucocorticoid sensitivity, resulting in significantly increased transactivating capacity (Russcher *et al*, 2005)		↑			✓
	• G/A (22 Glu →Glu) **[rs6189]** • G/A (23 Arg →Lys) **[rs6190]** Collectively known as ER22/23K polymorphism	3% 5%	• Lower CRP levels (van Rossum *et al*, 2004a) • Associated with a relative resistance to glucocorticoids (van Rossum *et al*, 2004b)	↓	↓	↑		
HSD11B1	• 83557 insA (intron 3) **[rs45487298]**	?	• Lower transcriptional activity with reporter gene assays (Draper *et al*, 2003)		↑			
	• T83597G (intron 3) **[rs12086634]**	25%	• Lower transcriptional activity with reporter gene assays (Draper *et al*, 2003) • Lower early morning cortisol levels and higher cortisol response to ACTH (Gambineri *et al*, 2006)		↓			
HSD11B2	• G-209A • G-126A	? ?	• Reduced promoter activity and affinity for activators nuclear factor 1 (NF1) and Sp1 resulting in diminished HSD11B2 transcription (Alikhani-Koupaei *et al*, 2007)		↓			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Muscle

Muscle atrophy is known to occur in cancer cachexia. This results from a depression of muscle protein synthesis, an increase in muscle protein degradation, or a combination of both (Eley & Tisdale, 2007). Experimental studies have also showed a shift in the myosin isoform content of skeletal muscle in cancer cachexia from type I to type II (Diffee *et al*, 2002). The following section examines the genetic variations that affect the structure and function of muscle as well as those that regulate muscle proteolysis.

Muscle structure and function

Genes involved in regulating muscle structure and function include ACTN3 (Alpha-actinin 3), which bind to actin at the Z-line within muscle fibres and act to anchor actin filaments, and IL15 (interleukin-15). IL-15 signals through IL-15 receptor alpha (IL-15RA) and is found in abundance in skeletal muscle. IL-15 has been shown to be anabolic, marked by an increase in myosin heavy chain accumulation (Quinn *et al*, 2002). Polymorphisms in ACTN3, IL15 and IL15RA are shown in table 4.8.

Steroid androgens play an important role in determining lean body mass and muscle strength. Variants in the gene coding for the androgen receptor (AR), which is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone and has been known to be capable of activating myogenic genes (Vlahopoulos *et al*, 2005), are also displayed in table 4.8.

Polymorphisms associated with alterations fat free mass in the gene encoding the vitamin D receptor (VDR) are also included in table 4.8.

Table S8 *Summary of polymorphisms regulating muscle structure and function*

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ACTN3	• C/T (577 Arg→TER) **[rs1815739]**	49%	-			↓		
VDR	• G/A **[rs1544410]**	44%	-		↑	↑		✓
	• T/C (1 Met→Thr) **[rs2228570]**	44%	• Greater transcriptional activation of reporter genes *in vitro* (Whitfield *et al*, 2001)			↓		
IL15	• C/T (intron1) **[rs1589241]**	29%	-		↑			
	• A/T (3' UTR) **[rs1057972]**	35%	-		↓	↑		
IL15RA	• A/C (182 Asn→Thr) **[rs2228059]**	45%	-			↕		
	• A/C (3' UTR) **[rs2296135]**	49%	-			↑		
AR	• Exon 1 CAG repeat polymorphism	?	• Length of the CAG repeat inversely related to transcription activity (Chamberlain *et al*, 1994; Choong *et al*, 1996)			*		

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies
*Men with ≥22 CAG repeats have significantly greater total fat free mass (Walsh *et al*, 2005)

Muscle proteolysis

Angiotensin converting enzyme (ACE) plays a critical role in the renin-angiotensin system by catalyzing the conversion of the inactive angiotensin I to angiotensin II, which is the physiologically active form of the hormone. Acute and chronic exposure to angiotensin II in animal models has been associated with weight loss and enhanced protein breakdown in skeletal muscle (Brink *et al*, 2001; Brink *et al*, 1996).

In atrophying muscles, the ubiquitin ligase, atrogin-1, is induced and this response is necessary for rapid atrophy. Foxo3 is known to act on the atrogin-1 promoter to cause atrogin-1 transcription and this leads to dramatic atrophy of myotubes and muscle fibres (Sandri *et al*, 2004). Polymorphisms in the ACE and FOXO3A gene are presented in table 4.9.

Table 4.9 Summary of polymorphisms regulating muscle proteolysis

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ACE	• Insertion/deletion (I/D) polymorphism Intron 16 (287bp) **[rs4646994]**	?	• I allele associated with lower ACE levels (Tiret *et al*, 1992)		↑	↑	↑	✓
FOXO3A	• C-1582T	?	-		↓			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Adipose tissue

Body-fat depletion is a major component of weight loss in cancer cachexia. Increased lipolysis appears to be a key factor underlying fat loss, though decreases in lipid deposition and adipocyte development may also contribute (Legaspi *et al*, 1987). The following section examines polymorphisms in genes regulating adipose tissue metabolism.

Adipogenesis

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. There are three subtypes of the receptor (PPARα, PPARβ and PPARγ). PPARα is most commonly expressed in organs and tissues in which fatty acid oxidation is active. PPARα is known to participate in the regulation of key proteins involved in extracellular lipid metabolism, fatty acid oxidation, and inflammation (Torra *et al*, 2001). PPARγ is an important regulator of fat cell function by orchestrating differentiation of new adipocytes and by inducing expression of genes promoting uptake of fatty acids, triglyceride synthesis and insulin sensitivity (Lehrke & Lazar, 2005). Polymophisms in the PPARA and PPARG gene are shown in table 4.10.

Also of interest are the lipin proteins (lipin-1, lipin-2, and lipin-3) which are thought to be required for glycerolipid biosynthesis. They also act as transcriptional coactivators that regulate expression of lipid metabolism genes (Reue, 2009). Variants of genes coding of lipin-1 (LPIN1) and lipin-2 (LPIN2) are also presented in table 4.10.

Table 4.10 Summary of polymorphisms regulating adipogenesis

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
PPARA	• C484G (162Leu→Val) **[rs1800206]**	4%	• Increased serum concentrations of total and LDL cholesterol (Tai *et al*, 2002) • Increased serum IL-6 in 162Val homozygotes (Skoczynska *et al*, 2005)	↑	↑			
PPARG	• C49G (12Pro→Ala) **[rs1801282]**	10%	• Lower fasting insulin and insulin resistance (Buzzetti *et al*, 2004; Meshkani *et al*, 2007) • Lower serum adiponectin levels (Takata *et al*, 2004; Yamamoto *et al*, 2002) • Lower mRNA expression of PI3-kinase and higher PPARG expression in men (Kolehmainen *et al*, 2003)		↑			✓
	• C161T (synonymous) **[rs3856806]**	13%	• Lower fasting insulin and insulin resistance (Moffett *et al*, 2005) • Increased plasma leptin (Meirhaeghe *et al*, 1998)		↑			
	• 115Pro→Gln **[rs1800571]**	<1%	• Defective phosphorylation at 114Ser leading to reduced ability to promote adipocyte differentiation (Ristow *et al*, 1998)		↑			
LPIN1	• A3333G **[rs2577262]** (Intron 13)	33%	-		*			
	• C181T **[rs2716609]** (Intron 18)	13%	• Higher insulin levels with C allele (Loos *et al*, 2007a)		↓			
LPIN2	• C/T **[rs3745012]**	?	-		↓			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies
* A/A homozygotes associated with higher resting metabolic rate (Loos *et al*, 2007a)

Lipid turnover and transport

During lipolysis, trigylcerides are broken down in a stepwise fashion to free fatty acids (FFAs). This process is partly modulated by the sympathetic nervous system induced secretion of cathecholamines which act though β1, β2 and β3 adrenergic receptors (β-ARs) (Large *et al*, 2004). The signals between β-adrenergic receptors and effector proteins are integrated by G proteins which are of composed an alpha, a beta, and a gamma subunit. Polymorphisms of genes coding for β-ARs and the beta subunit of the G proteins (GNB3) are shown in table 4.11.

Lipoprotein lipase (LPL) is an enzyme that hydrolyses lipids in lipoproteins and plays a central role in the overall lipid metabolism and transport (Mead *et al*, 2002). Fatty acid binding proteins (FABPs) are a family of carrier proteins for fatty acids and other lipophilic substances. These proteins are thought to facilitate the transfer of fatty acids between extra and intracellular membranes (Chmurzynska, 2006). Polymorphisms in the LPL and FABP genes are also shown in table 4.11.

Table 4.11 Summary of polymorphisms regulating lipid turnover and transport

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ADRB1	• 389Arg→Gly **[rs1801253]**	32%	• Diminished adenylyl cyclase activity (Mason *et al*, 1999) • Diminished β1-AR-G-protein interaction (Joseph *et al*, 2004)		↓			✓
	• 49Ser→Gly **[rs1801252]**	?	• Higher basal and agonist-stimulated adenylyl cyclase activity (Levin *et al*, 2002) • Higher desensitization to sustained stimulation (Levin *et al*, 2002)		↑			
ADRB2	• C-47T (-19Cys→Arg) (5' leader cistron) **[rs1042711]**	43%	• Greater β2-AR expression in -19Cys (McGraw *et al*, 1998)		↓			
	• C-20T **[rs1801704]**	48%	-		↓			
	• C491T (164Thr→Ile) **[rs1800888]**	1%	• Diminished β2-AR-G-protein interaction (Green *et al*, 1993) • Decreased native adipocyte receptor function (Hoffstedt *et al*, 2001)					
	• G46A (16Gly→Arg) **[rs1042713]**	36%	• 16Gly associated with increased insulin resistance (Masuo *et al*, 2005)		↓			✓
	• G79C (27Glu→Gln) **[rs1042714]**	47%	-		↓			✓
ADRB3	• 64Trp→Arg **[rs4994]**	9%	• Associated with lower lipolytic activity (Umekawa *et al*, 1999)		↑			✓

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
GNB3	• C825T (synonymous) **[rs5443]**	39%	• Associated with increased intracellular signal transduction (Siffert *et al*, 1998)		↑			
LPL	• 447 Ser→TER **[rs328]**	12%	• Significantly lower IL-8 levels (Ak *et al*, 2007) • Increased LPL activity (Groenemeijer *et al*, 1997; Kozaki *et al*, 1993)	↓	↓			✓
	• G-93T **[rs1800590]**	2%	-		↓			
	• G-53C	?	-		↓			
FABP1	• A340G (94 Thr→Ala) **[rs1801273]**	?	• Higher baseline free fatty acid (Brouillette *et al*, 2004)		↓			
FABP2	• 54 Ala→Thr	?	• Associated with higher levels of CRP and IL-6 (de Luis *et al*, 2007)	↑	↑			✓

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Adiopkines and adipokine receptors

Adipose tissue is also recognized as a major endocrine organ because the tissue synthesizes and secretes an array of protein hormones and signals (Fantuzzi, 2005). These adipokines act locally in an autocrine/paracrine manner and/or as endocrine signals to regulate appetite, energy expenditure and a range of physiological processes including insulin sensitivity and the inflammatory response which may have an important role in the pathogenesis of cancer cachexia (Kerem *et al*, 2008).

Resistin is an adipocyte-derived pro-inflammatory cytokine. Resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al*, 2006). There are three polymorphisms within the RETN gene which codes for resistin that may influence the development of cachexia. These are shown in table 4.12.

Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (Diez & Iglesias, 2003). Adiponectin binds to a number of receptors including adiponectin receptor 1 and 2. An increase in adiponectin concentration has been assoicated with cachexia in patients with heart failure (McEntegart *et al*, 2007). Leptin is a protein hormone secreted by adipose tissue that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. Leptin acts through the leptin receptor. Polymorphisms in genes coding for adiponectin, leptin and their respective receptors are also presented in table 4.12.

Table 4.12 Summary of adipokine and adipokine receptor polymorphisms

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
RETN	• C-420G **[rs1862513]**	35%	• Increased plasma resistin (Cho *et al*, 2004; Osawa *et al*, 2007)	↑	↑			✓
	• A-537C	?	• Increased plasma resistin with A allele (Cho *et al*, 2004)	↓	↓			
	• ATG repeat in 3'UTR **[rs11270887]** Allele 3 (6 repeats)	?	• Lower fasting insulin levels (Pizzuti *et al*, 2002) • Lower insulin resistance (Pizzuti *et al*, 2002)		↓			
ADIPOQ	• G-11391A **[rs17300539]**	7%	• Lower adiponectin levels with -11391GG and with -11377G allele (Buzzetti *et al*, 2007)			↑		
	• C-11377G **[rs266729]**	30%	• Lower adiponectin levels with -11391GG and with -11377G allele (Buzzetti *et al*, 2007)			↓		
	• G276T (intron 2) **[rs1501299]**	28%	• Increased adiponectin levels with T/T genotype (Mousavinasab *et al*, 2006; Qi *et al*, 2005) • Increased adiponectin mRNA levels in visceral fat with T allele (Fredriksson *et al*, 2006)		↓			✓
	• T45G (synonymous) **[rs2241766]**	?	• Increased plasma adiponectin (Berthier *et al*, 2005; Mackevics *et al*, 2006)		↓	↑		✓
	• A2019del	?	• Increased plasma adiponectin (Pollin *et al*, 2005)		↑			
ADIPOR1	• G-8503A **[rs666089]** • T-1927C	<1% ?	• Lower insulin sensitivity (Stefan *et al*, 2005)					

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ADIPOR2	• G795A **[rs16928751]** • C870A **[rs35854772]** • C963T **[rs9805042]** * all synonymous	12% ? 14%	• 795A, 870A, 963T haplotype associated with higher adiponectin levels (Broedl *et al*, 2006)		↓			
LEP	• G-2548A **[rs7799039]**	49%	• Higher leptin levels (Mammes *et al*, 1998)		↓			✓
	• Single nucleotide deletion codon 133	?	• Very low serum leptin levels (Montague *et al*, 1997)		↑			
LEPR	• A27265G (223Gln→Arg) **[rs1137101]**	47%	• Higher insulin, leptin levels with 223Gln allele (Guizar-Mendoza *et al*, 2005)		↓			✓
	• A5193G (109Lys→Arg) **[rs1137100]**	29%	• Decreased fasting insulin levels in postmenopausal women with impaired glucose tolerance (Wauters *et al*, 2001b) • Increased leptin levels in 109Lys homozygotes (Wauters *et al*, 2001a)		↓			✓
	• G44704C (656Lys→Asn) **[rs8179183]**	10%	• Decreased leptin levels in obese patients following lifestyle modification in 656Lys homozygotes (de Luis Roman *et al*, 2006) • Increased fasting insulin levels in postmenopausal women with impaired glucose tolerance (Wauters *et al*, 2001b)		↑			
	• T35861C (synonymous) **[rs3790419]**	20%	-		↓			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Appetite regulation

Anorexia, defined as the loss of the desire to eat, is common in patients with cancer cachexia. In addition to any effects of the tumour on the gastrointestinal tract and psychological depression, patients with cancer frequently have a decreased taste and smell of food (DeWys & Walters, 1975). Cancer anorexia may be a result of an imbalance between orexigenic signals and anorexigenic signals. The following section explores the variants in the genes encoding these signals.

Ghrelin is synthesized principally in the stomach and is released in response to acute and chronic changes in nutritional state. In addition to having a powerful effect on the secretion of growth hormone, ghrelin stimulates food intake and transduces signals to hypothalamic regulatory nuclei that control energy homeostasis (Hosoda *et al*, 2002). The melanocortin 4 receptor, a G-protein coupled receptor, binds α-melanocyte stimulating hormone (α-MSH). Melanocortin 4 receptors have been found to be involved in feeding behaviour and the regulation of metabolism (Fan *et al*, 1997). Endocannabinoids, acting at brain cannabinoid type 1 receptors, play a role in stimulating appetite and ingestive behaviours, partly through interactions with more established orexigenic and anorexigenic signals (Kirkham, 2005). Ciliary neurotrophic factor (CNTF) has been shown to activate hypothalamic leptin-like pathways which suppress food intake without triggering hunger signals or associated stress responses that are otherwise associated with food deprivation (Lambert *et al*, 2001). CNTF acts via CNTF receptors (CNTFRs) which are located in hypothalamic nuclei involved in feeding. Polymorphisms of interest in the genes encoding ghrelin (GHRL), melanocortin 4 receptor (MC4R), cannabinoid type 1 receptor (CNR1) and the CNTFR are shown in table 4.13.

Table 4.13 Summary of polymorphisms regulating appetite

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
GHRL	• C247A (72 Leu→Met) **[rs696217]**	9%	• Lower insulin secretion during glucose tolerance test (Korbonits *et al*, 2002)		↑			✓
	• G346A (51 Arg→Gln) **[rs34911341]**	?	• 51Gln allele associated with lower ghrelin concentrations (Poykko *et al*, 2003)		↑			
	• A-501C **[rs26802]**	37%	-		↓			
MC4R	• G307A (103 Val→ Ile) **[rs2229616]**	1%	• Lower serum triglyceride levels (Bronner *et al*, 2006)		↓			✓
	• A751C (251 Ile→Leu) **[rs52820871]**	?	-		↓			
CNR1	• G1422A (synonymous) **[rs1049353]**	23%	-		↑			
CNTFR	• C174T	?	-		↑			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Others

Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins. MTs are encoded by a family of genes consisting of 10 functional MT isoforms, and the encoded proteins are conventionally subdivided into 4 groups: MT-1, MT-2, MT-3, and MT-4 proteins. The physiological roles of MTs are not well understood but they are thought to play a role in the control of oxidative stress and protection against inflammation (Simpkins, 2000).

The P2Y-receptors belong to the superfamily of G-protein-coupled receptors and mediate the actions of extracellular nucleotides in cell-to-cell signalling. The P2Y11 receptor is highly expressed in immunocytes and may play a role in the differentiation of these cells (von Kugelgen, 2006).

Glutamine:fructose-6-phosphate aminotransferase (GFAT) is the rate-limiting enzyme of the hexosamine biosynthetic pathway (HBP), which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate. The flux through the HBP has been shown to be linked to the regulation of energy intake and energy expenditure (Obici *et al*, 2002).

Fat mass and obesity associated (FTO) is thought to play a role in energy homeostasis but its exact function is unknown.

Genetic variants encoding the proteins discussed above may play a role in the development of cachexia and are listed in table 4.14.

Table 4.14 Summary of other notable polymorphisms

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
MT1B	• C/T (intron) **[rs8052334]**	50%	-		↓			
MT2A	• A-209G **[rs1610216]**	25%	• A/A genotype associated with higher plasma levels of IL-6 (Giacconi *et al*, 2005)	↓				
	• C838G (3' UTR) **[rs10636]**	25%	• C allele associated with increased MCP-1 and decreased NK cell cytotoxicity (Giacconi *et al*, 2007)					
P2RY11	• 87 Ala→Thr **[rs3745601]**	10%	• Elevated levels of CRP (Amisten *et al*, 2007)	↑				
GFPT1	• G-913A	?	-		↓			
FTO	• A/T (intron 1) **[rs9939609]**	46%	-		↓			✓
	• T/C (intron 1) **[rs1421085]** • T/G (intron 1) **[rs17817449]**	46% 46%	-		↑			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Analysis of results

Out of the 184 polymorphisms that have been identified, the functional or clinical significance of only 42 polymorphisms have been verified in more than one study.

Of these 42 polymorphisms, 13 polymorphisms have been shown to have more than one effect on clinical features associated with cancer cachexia (i.e. inflammation, changes in lean and/or fat mass, and overall survival). These 13 polymorphisms represent the most promising candidates in terms of susceptibility biomarkers of cancer cachexia (Table 4.15) and are explored in more detail below.

The C allele of the A37674C *SELP* polymorphism (rs6136) is associated with decreased serum P-selectin levels (Miller *et al*, 2004; Volcik *et al*, 2006). P-selectin is required for efficient recruitment of neutrophils in acute inflammation and of macrophages in later stages of the inflammatory response and serum levels of P-selectin have been found to be significant prognostic factors in survival in patients with gastric and colorectal malignancies (Alexiou *et al*, 2001; Alexiou *et al*, 2003)

TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Within the *TNF* gene , the -308A allele (rs1800629) has been associated with an increased TNF-α production as well as a 6-fold increase in transcription of the TNF gene (Sallakci *et al*, 2005; Wilson *et al*, 1997). Interestingly, the A/A genotype has been linked to increased fat accumulation in women (Hoffstedt *et al*, 2000). The -863A allele (rs1800630) associated with decreased

transcriptional activity and reduced serum TNF-α levels (Day *et al*, 1998; Kaluza *et al*, 2000; Sharma *et al*, 2006; Skoog *et al*, 1999). Of note, obese individuals express 2.5-fold more TNF mRNA in fat tissue (Hotamisligil *et al*, 1995).

LTA, a member of the tumour necrosis factor family, is a cytokine produced by lymphocytes, and mediates a large variety of inflammatory and immunostimulatory, responses. The G allele of the 252 A>G polymorphism (rs909253) has been associated with increased serum TNF-α levels (McArthur *et al*, 2002; Stuber *et al*, 1996), and patients who are A/A homozygotes have been linked with better prognosis in lung cancer and gastric cancer (Shimura *et al*, 1994; Shimura *et al*, 1995).

IL-1β is a cytokine protein which is encoded by the *IL1B* gene and is an important mediator of the inflammatory response. The -31 C>T (rs1143627) and -511 C>T (rs16944) polymorphisms in the promoter region of the *IL1B* gene have been linked with increased transcriptional activity of the *IL1B* gene and subsequently increased IL-1β production (Wen *et al*, 2006). The -31C and -511T alleles are linked with poorer progression-free survival and overall survival in advanced gastric cancer (Graziano *et al*, 2005).Increased IL-1β levels have been linked to a synonymous C to T polymorphism at nucleotide position 3953 (rs1143634) (Hernandez-Guerrero *et al*, 2003). The T/T genotype has also been associated with lower plasma levels of IL-1 receptor antagonist (IL-1RA) (Tolusso *et al*, 2006). In addition the T allele has been found to be a major risk factor for cachexia in gastric cancer (Zhang *et al*, 2007), as well as being linked to lower total fat mass(Strandberg *et al*, 2006). The T/T genotype was found to be associated with shorter survival in pancreatic cancer (Barber *et al*, 2000).

IL-6 is a cytokine involved in a wide variety of biological functions. It is critical for B-cell differentiation and maturation, immunoglobulin secretion, cytotoxic T-cell differentiation and acute-phase protein production (Kishimoto, 2005). The -174 G>C promoter polymorphism (rs1800795) in the *IL6* gene has been associated with lower serum levels of IL-6 (Fishman *et al*, 1998). The G allele has been linked to higher fasting insulin and lower adiponectin levels which may have a role in the regulation of adiposity (Yang *et al*, 2005). In addition the C/C genotype has been associated with lower fat free mass and increased waist circumference (Berthier *et al*, 2003; Roth *et al*, 2003).

IGF-1 plays an important role in childhood growth and continues to have anabolic effects in adults. IGF-1 is one of the most potent natural activators of the Akt signalling pathway, a stimulator of cell growth and multiplication. IGF-1 also mediates many of the growth-promoting effects of growth hormone (GH) (Jones & Clemmons, 1995). A CA repeat polymorphism is found within the promoter region of the *IGF1* gene. The 19CA repeat allele is associated with both lower serum IGF-I levels and IGFBP-3 levels (Morimoto *et al*, 2005; Rosen *et al*, 1998). The 19CA repeat allele is also associated with reduced risk of weight gain (Landmann *et al*, 2006; Voorhoeve *et al*, 2006). Of note, increasing IGF-1 levels have been associated with poorer prognosis in oesophageal cancer patients (Sohda *et al*, 2004).

Angiotensin converting enzyme (ACE) plays a critical role in the renin-angiotensin system by catalyzing the conversion of the inactive angiotensin I to angiotensin II, which is the physiologically active form of the hormone. Acute and chronic exposure to angiotensin II in animal models has been associated with weight loss and enhanced protein breakdown in skeletal muscle (Brink *et al*, 2001; Brink *et al*, 1996). A common insertion/deletion (I/D) polymorphism (rs4646994) is present in intron 16 of the *ACE* gene. The I allele has been associated with lower circulating ACE levels (Tiret *et al*, 1992). The D allele has been

associated with increased strength gains thorough isometric training (Folland *et al*, 2000) and has also been associated with obesity (El-Hazmi & Warsy, 2003; Riera-Fortuny *et al*, 2005). The D/D genotype has been linked with increased survival in women with colorectal cancer (Rocken *et al*, 2007).

Lipoprotein lipase (LPL) is an enzyme that hydrolyses lipids in lipoproteins and plays a central role in the overall lipid metabolism and transport (Mead *et al*, 2002). The rs328 polymorphism in the LPL gene results in a premature stop codon at amino acid 447.The stop codon results in lower LPL activity (Groenemeijer *et al*, 1997; Kozaki *et al*, 1993), and is associated with lower levels of IL-8 (Ak *et al*, 2007). Individuals not in possession of the stop codon are associated with central obesity (Huang *et al*, 2006).

Resistin is an adipocyte-derived pro-inflammatory cytokine. Resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al*, 2006). The -420 C>G polymorphism (rs1862513) has been shown to be linked to increased plasma resistin (Cho *et al*, 2004; Osawa *et al*, 2007), and individuals with the G/G genotype have been associated with an increased prevalence of obesity (Norata *et al*, 2007). Overall, increased plasma resistin has been shown to correlate with increased CRP and insulin resistance (Degawa-Yamauchi *et al*, 2003; Kusminski *et al*, 2007; Nagaev *et al*, 2006; Osawa *et al*, 2007; Silswal *et al*, 2005).

Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (Diez & Iglesias, 2003). An increase in adiponectin concentration has been associated with cachexia in patients with heart failure (McEntegart *et al*, 2007). The *ADIPOQ*

gene, which codes for adiponectin, has a 45 T>G polymorphism (rs2241766) that is associated with increased plasma adiponectin (Berthier *et al*, 2005; Mackevics *et al*, 2006). Individuals with the G/G genotype have been observed to be leaner with less abdominal fat (Loos *et al*, 2007b).

Table 4.15 Polymorphisms replicated in more than one study and with at least two effects on clinical features associated with cancer cachexia (n=13)

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival
SELP	• A37674C (715 Thr→Pro) **[rs6136]**	9%	• Decreased serum P-selectin levels (Miller *et al*, 2004; Volcik *et al*, 2006)	↓			↑
TNF	• G-308A **[rs1800629]**	17%	• Increased TNF-α production (Sallakci *et al*, 2005) • Six fold increase in transcription of TNF-α (Wilson *et al*, 1997)	↑	↑		
	• C-863A **[rs1800630]**	15%	• Reduced total serum IgE levels (Sharma *et al*, 2006) • Reduced serum TNF-α levels (Sharma *et al*, 2006) • 31 % decrease in transcription of TNF-α (Skoog *et al*, 1999)	↓	↓		
LTA	• A252G (Intron 1) **[rs909253]**	36%	• Increased serum TNF-α levels (McArthur *et al*, 2002; Stuber *et al*, 1996)	↑			↓
IL1B	• C-31T **[rs1143627]**	36%	• Increased expression of IL-1β gene with T allele (Lind *et al*, 2007) • Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al*, 2006)	↑			↑
	• C-511T **[rs16944]**	36%	• Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al*, 2006) • No significant increase in IL-1β production in response to LPS in patients homozygous for T allele (Awomoyi *et al*, 2005)	↓			↓

Gene	Polymorphism	Minor Allele Frequency	Functional Significance	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival
IL1B	• C3953T (3954) (synonymous) **[rs1143634]**	21%	• T/T genotype associated with lower plasma levels of IL1-RA (Tolusso *et al*, 2006) • Increased human amniochorion IL-1β production after stimulation with LPS (Hernandez-Guerrero *et al*, 2003)	↑	↓		↓
IL6	• G-174C **[rs1800795]**	46%	• Lower levels of IL-6 in plasma in healthy subjects (Fishman *et al*, 1998) • Higher fasting plasma insulin levels with G allele (Yang *et al*, 2005) • Lower circulating adiponectin levels with G allele (Yang *et al*, 2005)	↓	↑	↓	
IGF1	• CA repeat promoter polymorphism 19 CA repeats (192bp)	?	• Lower serum IGF-I levels (Morimoto *et al*, 2005; Rosen *et al*, 1998) • Lower IGFBP-3 levels (Morimoto *et al*, 2005)		↓		↑
ACE	• Insertion/deletion (I/D) polymorphism Intron 16 (287bp) **[rs4646994]**	?	• I allele associated with lower ACE levels (Tiret *et al*, 1992)		↑	↑	↑
LPL	• 447 Ser→TER **[rs328]**	12%	• Significantly lower IL-8 levels (Ak *et al*, 2007) • Increased LPL activity (Groenemeijer *et al*, 1997; Kozaki *et al*, 1993)	↓	↓		
RETN	• C-420G **[rs1862513]**	35%	• Increased plasma resistin (Cho *et al*, 2004; Osawa *et al*, 2007)	↑	↑		
ADIPOQ	• T45G (synonymous) **[rs2241766]**	?	• Increased plasma adiponectin (Berthier *et al*, 2005; Mackevics *et al*, 2006)		↓	↑	

↑ Increase; ↓ Decrease

4.5 Discussion

Like many complex conditions and diseases, the risk of developing cancer cachexia is probably determined by multiple genetic factors and environmental factors are likely to add to the heterogeneity of the condition. Although the number of reports on polymorphic gene variants associated with multi-factorial diseases and conditions are dramatically growing, very few studies provide firm and reliable evidence of causative relationships between these polymorphisms and risk or pathogenesis. Indeed, in a recent review of allelic association with common disease phenotypes, only six of 166 associations subjected to multiple evaluations were confirmed consistently (Hirschhorn *et al*, 2002). Possible causes of false-positive association studies include population stratification, variable linkage disequilibrium and genotype misclassification. In addition, in many of these studies the possible effects of single gene variants were assessed in situations when combined impacts of multiple factors could be expected (Loktionov, 2003). In this chapter, 42 out of 184 polymorphisms have been identified with a potential role in the development of cachexia and have been independently verified in at least one repeat study.

It can be assumed that analysis of combinations of gene variants encoding interacting factors within a biological chain or cascade, rather than isolated investigation of its single components, may have more chances to reveal real causative connections between gene polymorphisms and phenotypes. In this chapter, functional polymorphisms in genes with a possible role in cachexia have been recorded as well as polymorphisms with clinical consequences related to cachexia such as inflammation, weight/body composition changes and cancer survival.

Of the 42 polymorphisms with a potential role in the development of cachexia that have been independently verified in at least one repeat study, 13 polymorphisms have been shown to

have more than one effect on clinical features associated with cancer cachexia. These 13 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers of cancer cachexia and should be further investigated.

In multigene studies, judicious selection of candidate genes and polymorphisms within them is a key element of study design. It is always important to choose genes, products of which interact within regulatory or metabolic pathways. In most cases it is not realistic to analyze all possible gene variants and combinations, hence existing polymorphisms should be initially prioritized on the basis of their likelihood to affect function of the encoded product (Tabor *et al*, 2002).

In conclusion, the present chapter has provided an initial framework in which to select and study the possible genetic variance associated with developing cancer cachexia by identifying polymorphisms with putative functional and/or clinical significance in relation to the development of cachexia. The identification of genetic variants that have undergone repeat studies allows the selection of robust and reliable candidates. Furthermore, prioritisation of the most likely genetic variants that are likely to influence the development of cachexia have been derived by selecting polymorphisms that influence two or more hallmarks of cachexia (i.e. systemic inflammation, loss of fat mass, loss of lean mass, reduced survival). Polymorphisms identified in this chapter will be studied in further detail for association with cancer cachexia in chapter 6.

CHAPTER 5

IDENTIFICATION OF NOVEL BIOMARKERS OF SKELETAL MUSCLE CANCER CACHEXIA: A TRANSCRITOMICS APPROACH

5.1 Summary

Aims

The aim of this chapter is to carry out global molecular profiling in the skeletal muscle of cancer patients and relate findings to percentage weight loss.

Methods

Rectus abdominis muscle biopsies were obtained from 18 upper gastrointestinal (UGI) cancer patients and 3 normal controls during surgery and RNA profiling was performed using the Affymetrix U133+2 platform.

Results

Quantitative significance analysis of microarrays produced an 83-gene signature that correlated with percentage weight loss. The most significant positive correlation was *CaMK2B* with weight loss. Previously postulated candidates genes involved in skeletal muscle wasting, the ubiquitin E3 ligases, were not related to weight loss in this human clinical study.

Conclusion

This chapter has potentially discovered new molecular biomarkers of human cancer cachexia. The *CaMK2B* gene correlated positively to weight-loss and excessive CaMKIIβ activation maybe a potential mechanism for reduced muscle protein synthesis in cachexia.

Polymorphisms in genes identified in this chapter may account for the inter-individual variation in developing cancer cachexia and will be further explored in Chapter 6.

5.2 Introduction

Cachectic patients suffer loss of both muscle mass and adipose tissue (with comparative sparing of visceral protein) and this tissue loss appears resistant to nutritional support (Nixon & Lawson, 1983). There is a need for more primary investigations to shed light on the detailed mechanisms that produce the syndrome in patients.

Muscle mass is maintained by physical activity, reflecting a balance between protein synthesis and degradation. Many of the molecular signalling pathways that are postulated to contribute to muscle atrophy in preclinical models mediate their effects through activation of the ubiquitin proteasome pathway (UPP) (Jagoe & Goldberg, 2001). Identification of two muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1, in a large number of animal models of atrophy (Bodine *et al*, 2001; Gomes *et al*, 2001) has been used to provide an argument for a major contribution of the UPP in muscle wasting, such that these genes are now measured as surrogate indicators of UPP activation .However, there is also evidence that the UPP is first activated with increasing weight loss then declines as the disease severity progresses (Khal *et al*, 2005). This suggests that UPP is a marker of protein turn-over rather than wasting *per se* (with turn-over increasing as the muscle weakens, but only while the patient continues to be ambulatory) or that UPP proteins are not reliable biomarkers. Furthermore, recent data indicates a dissociation between protein dynamics *in vivo* and activation or expression of the UPP-related signalling molecules in human skeletal muscle (Greenhaff *et al*, 2008). Overall, it is not clear what regulates muscle mass *in vivo*

nor is it clear to what extent protein degradation contributes over inhibition of protein synthesis (Emery *et al*, 1984; Greenhaff *et al*, 2008). Given the paucity of data derived from cancer cachexia patients, including study of the UPP system, this chapter sought to carry out global molecular profiling in the skeletal muscle of cancer patients and relate findings to clinical status (i.e. percentage weight loss).

5.3 Methods

Patients

Patients were recruited from surgical clinics from Royal Infirmary of Edinburgh as described in Chapter 2. Participating patients had a diagnosis of upper gastrointestinal cancer (oesophageal, gastric or pancreatic) and were undergoing surgery with the intent of resection of the primary tumour. A small number of weight stable patients undergoing surgery for benign, non-inflammatory conditions (n = 3) were also recruited for the study. Patient-reported height, weight, and weight history were collected. Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2). A weight loss ≥5% was used to identify weight-losing (WL) cancer patients as opposed to weight stable (WS) individuals.

Skeletal muscle biopsy

All biopsies were taken at the start of open abdominal surgery. The edge of *rectus abdominis* was exposed and a $1cm^3$ specimen removed using sharp dissection. The biopsy was snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Nuclear protein extraction and RNA isolation

Nuclear proteins were extracted from approximately 20mg of skeletal from which ribonucleic acid (RNA) was obtained from using commercially available kits. The methods are described in detail in Chapter 2.

Microarray target preparation and hybridisation

Total RNA (3.5 µg) was reverse transcribed and processed according to the protocol provided by Affymetrix Inc. for the GeneChip Expression 3' Amplification One-Cycle Target Labelling and Control Reagents kit (Affymetrix, High Wycombe, UK). Reverse transcription and second strand cDNA synthesis were followed by *in vitro* transcription and biotinylation. Biotinylated cRNA products were cleaned up using columns (Affymetrix). The quality of the biotinylated cRNA was assessed by Nanodrop (LabTech International, UK) and BioAnalyzer (Agilent Technologies) instruments and the cRNA was then fragmented according to Affymetrix protocols. Samples were hybridized to the HGU-133plus2 GeneChip array (covering approximately 54,000 sequences). The raw data files can be accessed at the Gene Expression Omnibus using the ID [GEO:GSE18832].

Microarray statistical analysis

Microarray data were analyzed using the Microarray Suite software (MAS) version 5.0 (Affymetrix). To improve the accuracy of the gene to probe relationship, a custom chip definition file (CDF) (Sandberg & Larsson, 2007) was used defining the Affymetrix probes by Ensembl transcript ID. Data were normalized using MAS5 and robust multi-array average (Irizarry *et al*, 2003). Genes called absent on every array by the MAS5 software were filtered from the data and remaining genes analysed using the quantitative function in significance analysis of microarrays (SAM) (Tusher *et al*, 2001) implemented in the Bioconductor suite (Gentleman *et al*, 2004). Percentage weight loss was used as the quantitative variable. To

test the robustness of the approach, the Limma package in the Bioconductor suite was used to identify genes covarying with weight loss. Both SAM and Limma generate a false discovery rate (FDR) (Benjamini *et al*, 2001). All genes identified by both procedures with an FDR <10% that covaried with weight loss were further examined.

5.4 Results

Twenty-one subjects were recruited (3 controls and 18 patients with upper gastrointestinal cancer). Patient demographics are shown in Table 5.1. Average weight loss of the cancer patients was 8.9% (range -0.5 to 43.8%). Compared with the control group, cancer patients had significant weight loss ($P < 0.01$) and had a lower BMI ($P = 0.08$). The controls were substantially younger ($P < 0.01$) and hence could not be used as a case control comparison group for the molecular profiling. Instead, gene expression was related to body mass status.

Table 5.1 Demographics of control and cancer patients

	Control (n = 3)	Cancer (n = 18)	*p*-value
Male / Female	2/1	12/6	N.A
Age (years) †	45 ± 2	67 ± 2	<0.01
% Weight Loss †	0	8.9 ± 1.6	<0.01
BMI †	28.5 ± 1.7	24.4 ± 0.8	0.08

Values are number of patients unless indicated otherwise; †Values are mean ± SE; BMI: body mass index.

Genes that varied with percentage weight loss were identified using the quantitative SAM methodology (Tusher *et al*, 2001). In this multiple comparison corrected correlation analysis, the weight stable group contained both cancer patients and three non-cancer controls in

order to identify *bona fide* cachexia associating genes. SAM identified 74 genes with a FDR between 0 and 10% (most <5% FDR) that covaried positively with weight loss (Table 5.2), and nine genes with a FDR between 0 and 10% (most <5% FDR) that covaried negatively with weight loss (Table 5.3). Correlation coefficients (R) for these 83 genes were generated using Pearson's product moment correlation. Positive coefficients ranged from 0.82 to 0.57 ($P < 0.01$), and for negatively correlating genes, R ranged from -0.74 to -0.65 ($P < 0.01$). Most of the genes correlating with weight loss had not been associated previously with cachexia in humans or animal models. Notably, the E3 ligases MURF1 and MAFbx were absent from this list.

Table 5.2 Genes positively correlated with weight loss (FDR <10%)

Ensembl Transcript ID	Gene	FDR %	R	Gene descriptions
ENST00000346990	CAMK2B	0	0.82	Calcium/calmodulin-dependent protein kinase type II beta chain
ENST00000291900	ZER1	0	0.77	Zyg-11 protein homolog (Zyg-11 homolog B-like).
ENST00000263974	TAF12	0	0.74	Transcription initiation factor TFIID subunit 12
ENST00000222214	GCDH	0	0.71	Glutaryl-CoA dehydrogenase
ENST00000329138	HGS	0	0.70	Hepatocyte growth factor-regulated tyrosine kinase substrate
ENST00000320548	GPS1	0	0.70	COP9 signalosome complex subunit 1
ENST00000259632	DCTN3	0	0.69	Dynactin subunit 3
ENST00000382538	TSC2	3.01	0.69	Tuberin (Tuberous sclerosis 2 protein).
ENST00000055077	RFC2	3.01	0.69	Replication factor C subunit 2
ENST00000378460	NUP160	5.03	0.68	Nuclear pore complex protein Nup160
ENST00000296456	APEH	3.01	0.68	Acylamino-acid-releasing enzyme
ENST00000261405	VWF	3.01	0.68	von Willebrand factor precursor (vWF)
ENST00000375865	LY6G5B	0	0.68	Lymphocyte antigen 6 complex
ENST00000287777	KBTBD5	0	0 67	Kelch repeat and BTB domain-containing proteir 5
ENST00000372475	TIE1	3 01	0 67	Tyrosine-protein kinase receptor Tie-1 precursor
ENST00000323013	27341	3.01	0.66	Gastric cancer antigen Zg14
ENST00000372928	TTC18	5.03	0.66	Tetratricopeptide repeat protein 18
ENST00000334029	PRODH	6.05	0.66	Proline oxidase
ENST00000355338	WARS	3.01	0.66	Tryptophanyl-tRNA synthetase
ENST00000306869	DCXR	3.01	0.65	L-xylulose reductase

Ensembl Transcript ID	Gene	FDR %	R	Gene descriptions
ENST00000383433	CSNK2B	5.03	0.65	Casein kinase II subunit beta
ENST00000344592	FLYWCH1	5.03	0.65	FLYWCH-type zinc finger 1 isoform a
ENST00000321265	NUDC	3.47	0.65	Nuclear migration protein nudC
ENST00000357720	TRMT1	5.03	0.65	TRM1 tRNA methyltransferase 1 homolog
ENST00000263436	PRPF31	6.05	0.65	U4/U6 small nuclear ribonucleoprotein Prp31
ENST00000354849	INTS4	5.03	0.65	Integrator complex subunit 4 (Int4).
ENST00000359512	FAM39E	5.03	0.65	FAM39B protein
ENST00000379795	25851	5.03	0.64	-
ENST00000322176	DNPEP	6.05	0.64	Aspartyl aminopeptidase
ENST00000287624	RPUSD3	5.03	0.64	RNA pseudouridylate synthase domain containing 3
ENST00000373909	RPA2	3.01	0.64	Replication protein A 32 kDa subunit
ENST00000373586	EIF3I	5.03	0.64	Eukaryotic translation initiation factor 3 subunit 2
ENST00000276671	C11orf59	5.03	0.64	UPF0404 protein C11orf59.
ENST00000337761	CORO6	3.01	0.64	Coronin-6.
ENST00000297183	EIF4EBP3	5.03	0.64	Eukaryotic translation initiation factor 4E-binding protein 3
ENST00000331483	P4HB	5.03	0.63	Protein disulfide-isomerase precursor
ENST00000290429	MCAT	5.03	0.63	Malonyl CoA-acyl carrier protein transacylase
ENST00000378319	Hs.461200	5.03	0.63	Protein FAM39A
ENST00000376901	YIF1A	5.03	0.63	Protein YIF1A (YIP1-interacting factor homolog A)

Ensembl Transcript ID	Gene	FDR %	R	Gene descriptions
ENST00000316940	PYCR2	5.03	0.63	Pyrroline-5-carboxylate reductase 2
ENST00000222388	ABCF2	5.03	0.63	ATP-binding cassette sub-family F member 2
ENST00000312536	C11orf68	6.05	0.63	Uncharacterized protein C11orf68 (Basophilic leukemia-expressed protein Bles03)
ENST00000372969	NDUFS5	5.03	0.63	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5
ENST00000301021	TRAPPC2L	6.05	0.62	*trafficking protein particle complex 2-like*
ENST00000331285	PCYT2	5.03	0.62	Ethanolamine-phosphate cytidylyltransferase
ENST00000191018	CTSA	5.03	0.62	Lysosomal protective protein precursor
ENST00000324366	PRMT5	5.03	0.62	Protein arginine N-methyltransferase 5
ENST00000310417	HHATL	5.03	0.62	Glycerol uptake/transporter homolog
ENST00000343043	DAP3	5.03	0.62	Mitochondrial 28S ribosomal protein S29
ENST00000263035	DHTKD1	5.03	0.62	dehydrogenase E1 and transketolase domain containing protein 1
ENST00000372195	RAPGEF1	5.03	0.61	Rap guanine nucleotide exchange factor 1
ENST00000039989	TTC17	5.03	0.61	Tetratricopeptide repeat protein 17
ENST00000263579	DCPS	5.03	0.61	Scavenger mRNA-decapping enzyme DcpS
ENST00000259239	IMP4	5.03	0.61	U3 small nucleolar ribonucleoprotein protein IMP4]
ENST00000306061	MT1E	5.03	0.60	Metallothionein-1E (MT-1E)
ENST00000354193	Hs.349096	5.03	0.60	Small VCP/p97-interacting protein.
ENST00000215591	POLRMT	5.03	0.60	DNA-directed RNA polymerase
ENST00000362082	MCM7	5.03	0.60	DNA replication licensing factor MCM7
ENST00000232014	BCL6	5.03	0.60	B-cell lymphoma 6 protein

Ensembl Transcript ID	Gene	FDR %	R	Gene descriptions
ENST00000330546	NM_182905	5.03	0.60	family with sequence similarity 39
ENST00000285718	FAM39B	5.03	0.60	Protein FAM39B
ENST00000262764	PGS1	5.03	0.60	phosphatidylglycerophosphate synthase 1
ENST00000371648	EDF1	5.03	0.60	Endothelial differentiation-related factor 1
ENST00000263378	AKAP8L	5.03	0.59	A-kinase anchor protein 8-like
ENST00000196968	GSTP1	5.03	0.59	Glutathione S-transferase P
ENST00000335781	PSPC1	5.03	0.59	paraspeckle protein 1
ENST00000369842	EMD	5.03	0.58	Emerin
ENST00000357500	POP5	5.03	0.58	Ribonuclease P/MRP protein subunit POP5
ENST00000373159	TRAPPC3	5.03	0.58	Trafficking protein particle complex subunit 3
ENST00000223073	RBM28	5.03	0.58	RNA-binding protein 28
ENST00000372622	DNTTIP1	5.03	0.58	Terminal deoxynucleotidyltransferase-interacting factor 1
ENST00000337392	CLPTM1	5.03	0.57	Cleft lip and palate transmembrane protein 1
ENST00000301264	DAPK3	5.03	0.57	Death-associated protein kinase 3
ENST00000240727	ZSCAN18	5.03	0.57	Zinc finger and SCAN domain-containing protein 18

FDR: false discovery rate; R: Pearson's correlation coefficient

Table 5.3 Genes negatively correlated with weight loss (FDR<10%)

Ensembl Transcript ID	Gene	FDR %	R	Gene descriptions
ENST00000272928	CXCR7	9.95	-0.65	C-X-C chemokine receptor type 7
ENST00000274643	SGK_85	3.01	-0.65	Uncharacterized serine/threonine-protein kinase
ENST00000310271	ALAS1	9.95	-0.65	5-aminolevulinate synthase
ENST00000217131	CTSZ	9.95	-0.66	Cathepsin Z precursor
ENST00000367858	SGK	3.47	-0.68	Serine/threonine-protein kinase Sgk1
ENST00000361303	C18orf1	3.01	-0.69	Uncharacterized protein C18orf1
ENST00000356438	HYLS1	9.95	-0.71	Hydrolethalus syndrome protein 1
ENST00000360110	TFRC	0	-0.72	Transferrin receptor protein 1
ENST00000355285	APCDD1	0	-0.74	Protein APCDD1 precursor

FDR: false discovery rate; R: Pearson's correlation coefficient

5.5 *Discussion*

Cancer cachexia is thought to arise due to an imbalance of the anabolic and catabolic pathways with consequent loss of muscle mass (along with an accompanying loss of adipose tissue). In the present study, the expression of 74 genes correlated positively with weight loss in cancer cachexia subjects and that of 9 correlated negatively with it.

The most significantly positively correlated gene with weight loss, CAMK2B, encodes calcium/calmodulin-dependent protein kinase II beta (CaMKIIβ), a serine/threonine protein kinase that is activated by Ca^{2+}/calmodulin, leading to autophosphorylation and maintenance of CaMKII activity even after the Ca^{2+} signal has diminished (Chin, 2004). CaMKIIβ is expressed in skeletal muscle. Levels of the protein as well as its phosphorylation status and activity increase after exercise training (Rose *et al*, 2007). In addition, it has recently been demonstrated that Ca(2+)-CaM-eEF2K signaling may be responsible for acute exercise-induced inhibition of muscle protein synthesis (van Hoek *et al*, 2009) and it is thus conceivable that chronic inappropriate activation of this 'endurance training'-related signaling molecule (Timmons *et al*, 2005) subdues normal maintenance of skeletal muscle mass. Additional factors that could modulate CaMKII activity include alterations in lipid metabolism (Raney & Turcotte, 2008). Finally, CaMKII has also been implicated in muscle adaptation through phosphorylation of HDAC5 leading to MyoD/MEF2-driven differentiation of muscle cells (McKinsey *et al*, 2000). CAMK2B may thus have an important role in regulating skeletal muscle function and metabolism.

Contrary to evidence from animal models (Bodine *et al*, 2001; Gomes *et al*, 2001; Mammucari *et al*, 2007), there were no significant differences in expression of the E3 ligases MURF1 and MAFbx. Given the robust increase in expression of the E3 ligases reported previously in various animal models of cachexia (Bodine *et al*, 2001; Gomes *et al*, 2001;

Lecker *et al*, 2004), it is somewhat surprising that the microarray did not detect any regulation of MuRF1 and MAFbx.

Previous results of E3 ligase expression analysis from other human models of cachexia have been contradictory. Studies including patients following bed rest, amputation for vascular disease, limb immobilization, chronic obstructive pulmonary disease, amyotrophic lateral sclerosis and ageing have demonstrated both increased and decreased expression of MuRF1 and MAFBx (de Palma *et al*, 2008; Doucet *et al*, 2007; Edstrom *et al*, 2006; Leger *et al*, 2006; Salanova *et al*, 2008). This seems to suggest that the ubiquitin E3 ligases do not play the same role in human cancer cachexia as that previously demonstrated in animal and cell studies. However, cancer cachexia encompasses a spectrum progressing from early weight loss through to severe muscle wasting. The prominence of the individual proteolytic pathways at different time points along this spectrum is yet to be determined and one must keep in mind that during severe tissue wasting, both breakdown (and of course synthesis) may well be reduced with the net balance between the two widened.

In an animal model of muscle atrophy the largest changes in E3 ligase expression were seen early, when rates of muscle weight loss were the highest, then fell as the rate of atrophy slowed. Nearly all the atrogene mRNAs returned to basal levels by 14 days while some atrogenes maintained their differential expression for a longer period (Sacheck *et al*, 2007). It may be likely that the patients in this study were recruited at a time point in which the expression of E3 ligases has returned to their baseline state.

A limitation of the current study is that the focus was on changes in total body mass which does not reveal the relative contributions from lean body mass and adipose tissue. The muscle gene expression results indicate, however, that there is a skeletal muscle molecular signature that reflects changes in whole body mass and it is hard to conceive that this is not somehow reflecting the changes in the muscle tissue.

In conclusion, the present chapter has shown a variety of genes which are correlated with weight loss in cancer. In particular, the up regulation of the CAMK2B gene appears to be a promising new biomarker for cancer cachexia. Polymorphisms in the genes identified in this chapter may play a role in the inter-individual variability of developing cachexia and will be examined in Chapter 6.

CHAPTER 6

A P-SELECTIN POLYMORPHISM IS ASSOCIATED WITH THE DEVELOPMENT OF CANCER CACHEXIA: RESULTS OF A CANDIDATE GENE ASSOCIATION STUDY

6.1 Summary

Aims

The aim of this chapter is to utilise a candidate gene approach to evaluate the association between genetic polymorphisms and the risks of developing cachexia in patients recruited across three centres with attempted validation using patients recruited from a fourth centre.

Methods

129 SNPs in 80 genes were analysed for association with cachexia based on degree of weight loss (>5, >10, >15%) alone or with the presence of systemic inflammation (CRP >10mg/l). 775 cancer patients from three centres were recruited at first presentation to a surgical or oncology clinic. Percentage weight loss was calculated from weight measured at recruitment and pre-morbid weight recalled. A validation association study was performed on an independently recruited cohort (n=101) of cancer patients from one centre. Association testing was adjusted for age, sex, tumour type, BMI and stage.

Results

21 SNPs had significant associations with cachexia phenotypes. However, only the C allele (minor allele frequency 10.7%) of the rs6136 (SELP) SNP was found to be associated with weight loss >10% both in the main study (OR 0.52, 95%C.I 0.29 – 0.93, p=0.026) and the validation study (OR 0.09, 95%C.I 0.01 – 0.98, p=0.035). Gene-group analysis was performed based on functional similarity according to gene ontology in the main study patients. Gene groups regulating appetite, glucocorticoid signalling, and mitogen activated

protein kinases (MAPK) activity were associated with both weight loss >15% and CRP >10mg/l (p=0.0008, p=0.018, and p=0.026 respectively)

Conclusion

The C-allele of the rs6136 polymorphism is associated with reduced risk of developing cachexia and this finding may prove useful in the risk stratification of pre-cachetic cancer patients. Gene groups regulating appetite, glucocorticoid signalling, and mitogen activated protein kinases (MAPK) activity may have central roles in the pathogenesis of cancer cachexia.

6.2 Introduction

Cachexia is particularly problematic in cancer, typified by poor prognosis and often associated with a lower response to chemotherapy and radiotherapy than might be expected (Tisdale, 2002). Patients are also more likely to report decreased quality of life (QoL) scores (Fearon *et al*, 2006). More than half of cancer patients suffer from cachexia, and it is responsible for death in up to 20% of cases (Tisdale, 2002). Cachexia is therefore a significant cause of morbidity and mortality in cancer patients.

Cachexia in its advanced phase (where patients may have lost 20-30% of their body weight) is easily identified. However, at this stage, the primary initiating events are frequently compounded by secondary factors (e.g. prolonged patient bed rest), and it is often impossible to attempt any realistic form of intervention, either practical or (given the patient's almost imminent demise) ethically advisable. Thus, one systematic approach to the

treatment of cachexia requires early identification of patients at risk of cachexia and the institution of prophylactic measures to attenuate its progression.

Based on our current knowledge of demographic and clinical factors, we are unable to predict, for any given cohort of patients, who will develop cancer cachexia and who will not. Such variation may, in part, be due to the patient's genotype. The cachexia syndrome is thought to result from a complex interaction between host neuroendocrine and cytokine systems and tumour-derived products (Skipworth *et al*, 2007). The wealth of known genetic polymorphisms in genes controlling pro/anti-inflammatory pathways, neuronal melanocortin signalling pathways, and muscle and adipose tissue metabolic pathways suggest their exploitable potential as biomarkers of inter-individual predictability of developing cachexia.

This aims of this chapter is to utilise a candidate gene approach to evaluate the association between genetic polymorphisms and the risks of developing cachexia in patients recruited across three centres, with attempted validation using patients recruited from a fourth centre.

6.3 Methods

Main study population

Study subjects were recruited from three centres from 2004 - 2008: NHS Lothian, UK; Cross Cancer Institute, Edmonton, Canada; and McGill University Health Centre, Montreal, Canada.

All patients with proven cancer diagnosis were considered and recruitment was conducted at first presentation to surgical or oncology clinics at each institution. Recruitment was performed sequentially with the following exclusion criteria: i) under 18 years of age; ii) learning disability, and mental health problems; iii) inability to give written, informed consent.

Overall, 855 patients with confirmed diagnosis of cancer were recruited. More than 98% of the study subjects were of European descent. Information collected on each patient included date of birth, date of diagnosis, type and stage of cancer. All patients underwent measurements of height and weight at the time of recruitment to the study. Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. There is evidence to support the reliability of self-reported weight and weight history (Perry *et al*, 1995; Stunkard & Albaum, 1981). Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost. Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2).

Where available, serum C-reactive protein (CRP) concentration was measured with an automated immunoturbidimetric assay by each institution's clinical chemistry department using blood collected from patients at the time of recruitment and before any therapeutic intervention.

Stage of disease was based on the American Joint Committee on Cancer stage groupings I, II, III and IV.

All subjects recruited had participated in clinical or research studies at the host institutions under ethically approved protocols. All patients provided written informed consent to allow analysis of their DNA.

Phenotype definitions

There is currently no consensus on the definition of cachexia. Many studies have used varying thresholds of weight loss to study cancer cachexia, the most common being >5% weight loss (Fox *et al*, 2009; Knoll *et al*, 2008; Maltoni *et al*, 2001) and >10% weight loss (Gordon *et al*, 2005; Skipworth *et al*; Zhang *et al*, 2007). A weight loss of >15% has been liked to major complications in cancer patients undergoing surgery (Antoun *et al*, 2009). A recent article by Evans et al. has suggested classifying the degree of cachexia as mild, moderate or severe, depending on whether the observed weight loss is >5%, >10% or >15%, respectively (Evans *et al*, 2008).

The presence of systemic inflammation (serum C-reactive protein (CRP) >10mg/l) has been linked to decreased survival in cancer patients (Mahmoud & Rivera, 2002; McMillan *et al*, 2003), and has also been correlated positively with weight loss in human cancer patients (Deans *et al*, 2009b; O'Gorman *et al*, 1999). CRP has also been incorporated into a three-factor mathematical definition of cachexia for patients with pancreatic cancer (Fearon *et al*, 2006). The latter multi-profile definition was found to have more prognostic value compared with weight loss alone.

To take into account the above, cachexia was classified based on degree of weight loss (>5, >10>, >15%), as well as weight loss in the presence of systemic inflammation.

Candidate gene and SNP selection

Candidate gene and SNP selection was based on a systematic literature review of SNPs with either putative functional or clinical relevance in the development of cancer cachexia described in Chapter 4. All 184 SNPs across 92 genes identified were considered for the study with priority given to the 13 polymorphisms which have been shown to have more than one effect on clinical features associated with cancer cachexia, and have been independently verified in at least one repeat study. SNPs with unknown rs numbers were excluded from the study.

In addition, all genes that were negatively correlated with weight loss and genes with a positive correlation of r >0.65 with weight loss identified in the gene expression analysis array study on muscle samples of cancer patients with cachexia described in Chapter 5 were subject to further study. From these genes, SNPs were selected using the following criteria:. i) within exons, ii) non-synomymous and, iii) with a MAF of >0.1 using the SNPbrowser software. This approach was chosen as it was not realistic to analyse all possible gene variants and combinations; SNPs selected in this manner were more likely to be functional variants and hence have a greater possibility to be associated with cachexia.

SNPs were entered into the Applied Biosystems online SNPlex assay design submission tool using their respective rs numbers. For SNPs that failed the SNPlex design pipeline, replacement SNPs were selected based on linkage disequilibrium (r^2 of at least 0.9) using the SNPbrowser software.

Overall 191 SNPs in 99 genes were considered for the association study (see table 6.2).

DNA extraction

Genomic DNA was extracted from whole blood using commercially available kits. The methods are described in detail in Chapter 2.

Genotyping

The Applied Biosystems SNPlex™ Genotyping System (Applied Biosystems, California, USA) was employed for SNP genotyping. All DNA samples were processed and assayed without regard to phenotype. DNA samples were separated electrophoretically on a 3730 DNA Genetic Analyzer (Applied Biosystems, California, USA), and automated allele calls and genotype clustering of each individual sample was performed by Applied Biosystems' GeneMapper® Software (version 4.0). All automatic calls by the software were evaluated by one researcher. Any SNPs with less than 90% of the sample auto-called by the software were either rescored manually or discarded if clustering confidence was low. Reproducibility was determined by rerunning entire plates of DNA samples and a reproducibility rate of 99.7% was achieved.

Individual samples were removed if more than 10% of SNPs failed genotyping, and individual SNPs were removed if more than 10% of samples failed. As an additional genotyping quality-control check, SNPs with significant deviation from Hardy-Weinberg equilibrium (HWE) ($p < 0.01$) were removed from the final analysis. SNPs with a MAF <0.03 were also removed from the final analysis.

Power calculations

Power calculations were performed using Quanto. For the most prevalent cachexia phenotype (i.e. >5% weight loss, 54% affected), the present study has between 43% to 97% power to detect an odds ratio of 1.5 for SNPs with a MAF of 0.05 – 0.35.

For the least prevalent cachexia phenotype (i.e. >15% weight loss & CRP >10 mg/l, 14% affected), the present study has between 12% to 40% power to detect an odds ratio of 1.5 for SNPs with a MAF of 0.05 – 0.35.

Statistical analysis

Statistical analyses were performed using PLINK (version 1.06) (Purcell *et al*, 2007). Patients who met the criteria for each of the proposed cachexia phenotypes were compared with patients who have lost ≤5% body weight as control. Unconditional logistic regression was employed to calculate odds ratios (OR) and their 95% confidence intervals (95% CI) for the minor allele of individual SNPs and its association with each proposed cachexia phenotype. All analyses were adjusted for covariates that may affect weight loss, i.e. age at diagnosis, sex, pre-morbid BMI, tumour type and stage.

To account for multiple testing, permutation testing was performed by running the adaptive permutation test in PLINK within each proposed phenotype. Permutation tests are often employed to adjust groups of correlated tests for multiple testing, since conventional methods such as Bonferroni correction are overly conservative when tests are correlated (Conneely & Boehnke, 2007). The adaptive permutation test in PLINK gives up permuting SNPs that are clearly going to be non-significant. This greatly speeds up the permutation

procedure, as SNPs that are not significant will drop out quite quickly, making it possible to properly evaluate significance for the handful of SNPs that require millions of permutations.

SNPs with a permuted p value of <0.02 within the same chromosomal region (within 10,000kb) were then analysed for any possible haplotype associations. Only haplotypes that had a frequency greater than 5% were considered for further analysis. Each identified haplotype and significant SNPs were then tested for association with percentage weight loss as a continuous variable.

Finally, candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology using AmiGO (Table 6.1). The set-based test in PLINK was used to analyse association between grouped SNPs and cachexia phenotypes. The set-based test selects the best set of SNPs whose mean of these single SNP statistics is significant after permutation, which is particularly suited to large-scale candidate gene studies (Ott & Hoh, 2003). The empirical p values of the set-based test were obtained by a permutation of 10,000 times of phenotype labels.

Table 6.1 Candidate genes groupings based on known functional similarity according to gene ontology

Regulation of MAPK activity		
GHRL	IL1B	TNF
TGFB2	ADRB2	IGF2
IL6		
Regulation of protein amino acid phosphorylation		
IL1B	TNF	CAMK2B
IGFBP3		
Inflammatory response		
IL6	CRP	IL10
IL13	IL1A	IL1B
NFKB1	TNF	ADIPOQ
GHRL	TNFRSF1A	AGER
Signal Transduction		
CAMK2B	IGF1	PPARG
GDF15	TLR4	IFNG
IL13	IFNGR1	LEPR
IL1B	TLR2	MIF
IRS1	CD14	LTA
NR3C1	AGER	IGFBP3
Immune response		
IL10	TLR4	IL18
TNF	IL4	IL6
IL12B	PPARG	TGFB2
Lipid metabolism		
PPARA	ADIPOR2	UCP3
PPARG	LPL	NFKB1
CRP	NFKBIA	IL1B
TNF	IRS1	
Proton transport		
UCP1	UCP2	UCP3
Protein metabolism		
NFKB1	IGF1	ADIPOQ
NFKBIA	IFNG	IL1B
TNF	IL10	CTSZ
ACE	TSC2	
Cell adhesion		
IL18	VCAM1	ADIPOQ
TGFB2	TNF	IL12B
ICAM1	SELP	
Regulation of calcidiol 1-monooxygenase activity		
IFNG	IL1B	TNF
Regulation of NF-kappaB activity		
IL1B	TLR4	TLR2
IL6	TLR5	LITAF
TNF	IL10	TNFRSF1A
NFKBIA	ADIPOQ	

Regulation of chemokine production		
IL6	TNF	IL18
Glucocorticoid signalling		
IL10	IL6	TNF
GHRL		
Insulin signalling		
GHRL	FOXC2	PPARG
IGF2	IGF1	IFNG
IRS1	TSC2	IL1B
TNF		
Regulation of phosphoinositide 3-kinase cascade		
TGFB2	TSC2	IGF1
Appetite		
GHRL	LEP	

Validation study

Subjects from the validation study were recruited from an independent centre, Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland from 2007 - 2008. Like the main study, all patients with proven cancer diagnosis were considered. Patients were recruited sequentially at first presentation to the oncology clinic. Exclusion criteria were identical to the main study.

In total, 101 cancer patients were recruited, all of whom were of European descent. Like the main study, all patients underwent measurements of height and weight at the time of recruitment. Premorbid weight was recalled by the patient and verified where possible from the medical notes. Individual weight loss was calculated and expressed as percentage of pre-illness body weight lost. Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2). Serum C-reactive protein (CRP) concentration was measured with an automated immunoturbidimetric assay at the institution's clinical chemistry department using blood collected from patients at the time of recruitment and before any therapeutic intervention.

Patients were genotyped for SNPs found to have permuted p <0.05 in the main study and quality control checks were carried out as described previously. As with the main study, patients in each of the proposed cachexia phenotypes were compared with patients with ≤5% weight loss as control, and association analyses were adjusted for age at diagnosis, sex, pre-morbid BMI, tumour type and stage.

6.4 Results

Following the relevant quality control checks, 129 SNPs in 80 genes (Table 6.2) were available for analysis in 775 patients. The overall completion rate of genotyping was 95.6%.

Table 6.2 Details of SNPs analysed

CHR	GENE	SNP	BP	A1	A2	HWE	MAF	CALL RATE
1	TNFRSF1B	rs496888	12155392	G	A	0.7342	0.3152	0.95262
1	TNFRSF1B	rs976881	12156340	A	G	0.1276	0.3158	0.95903
1	TNFRSF1B	rs1061622	12175541	G	T	0.5976	0.2252	0.94366
1	TNFRSF1B	rs3397	12189878	C	T	0.9382	0.3777	0.96287
1	TNFRSF1B	rs1061631	12191085	A	G	0.5167	0.2133	0.97567
1	TAF12	rs3795845	28804211	C	A	1	0.2744	0.8633
1	TAF12	rs1804642	28821087		G	N.A	0	0.92958
1	DIO1	rs11206244	54148288	T	C	0.562	0.33	0.94878
1	DIO1	rs11206246	54150613	C	T	0.22	0.09921	0.96799
1	LEPR	rs1137100	65809028	G	A	0.414	0.2769	0.96415
1	LEPR	rs1137101	65831100	G	A	0.7712	0.4519	0.98464
1	LEPR	rs1805134	65839696	C	T	0.0651	0.1985	0.8322
1	LEPR	rs8179183	65848539	C	G	1	0.1706	0.97951
1	VCAM1	rs3176860	100959806	G	A	0.6359	0.3748	0.93086
1	CRP	rs2794520	157945439	T	C	0.4986	0.3242	0.93214
1	CRP	rs1130864	157949714	T	C	0.1479	0.3143	0.75588
1	CRP	rs1800947	157950061	C	G	1	0.05867	0.96031
1	SELP	rs6136	167830574	C	A	0.3419	0.1073	0.96031
1	IL10	rs1800872	205013029	A	C	0.2993	0.2338	0.9283
1	IL10	rs1800896	205013519	G	A	0.4675	0.4704	0.97311
1	HSD11B1	rs12086634	207946881	G	T	1	0.2036	0.95903
1	HSD11B1	rs2236903	207950102	T	A	0.7536	0.2286	0.9411
1	TGFB2	rs947712	216631503	A	G	0.002483	0.3503	0.9283
1	TGFB2	rs1890995	216671300	T	C	0.7866	0.2868	0.93982
1	TGFB2	rs1418553	216676876	T	C	0.7955	0.298	0.97951
1	TLR5	rs5744168	221351822	T	C	1	0.06216	0.9475
2	LTBP1	rs817529	33350838	G	A	0.5945	0.3921	0.97311
2	IL1R1	rs2228139	102148080	G	C	0.7606	0.06425	0.8968
2	IL1A	rs17561	113253693	T	G	0.2042	0.2748	0.98079
2	IL1A	rs1800587	113259430	T	C	0.2043	0.2793	0.96287
2	IL1B	rs1143634	113306860	T	C	0.4445	0.2137	0.94366
2	IL1B	rs1143627	113310857	C	T	0.9356	0.3406	0.97183
2	IL1B	rs16944	113311337	A	G	0.5209	0.3618	0.7132
2	IRS1	rs1025333	227353768	A	T	0.3098	0.08016	0.94238
2	IRS1	rs2234931	227370996	A	G	0.3025	0.0748	0.97567
2	CXCR7	rs10183022	237146707	A	G	0.2562	0.4379	0.5877
2	CXCR7	rs9287599	237153091	G	A	0.05299	0.07486	0.93214
2	CXCR7	rs1045879	237154642	T	C	0.07954	0.2925	0.97183
3	GHRL	rs35681	10304376	A	G	0.5556	0.4744	0.94878
3	GHRL	rs42451	10305376	T	C	0.9248	0.2661	0.93598
3	GHRL	rs696217	10306456	T	G	0.04991	0.07397	0.9347
3	GHRL	rs34911341	10306518	T	C	1	0.006711	0.95391
3	GHRL	rs26802	10307364	G	T	0.06909	0.3233	0.8912
3	PPARG	rs1801282	12368124	G	C	1	0.1265	0.95647
3	PPARG	rs1800571	12397847		C	N.A	0	0.95391
3	PPARG	rs3856806	12450556	T	C	0.5231	0.1341	0.93086
3	KBTBD5	rs6805421	42703147	A	G	0.149	0.3686	0.92061
3	KBTBD5	rs123509	42708471	A	G	0.09578	0.2496	0.8412
3	KBTBD5	rs3846062	42708670	T	G	0.008731	0.4232	0.95903
3	APEH	rs4855881	49690449	C	T	0.5131	0.4494	0.9872
3	APEH	rs2960548	49695396	G	C	0.3798	0.4539	0.97183
3	ADIPOQ	rs17300539	188042153			N.A	N.A	0
3	ADIPOQ	rs266729	188042167	G	C	0.2185	0.229	0.97567
3	ADIPOQ	rs2241766	188053585	G	T	0.8719	0.1313	0.9411

CHR	GENE	SNP	BP	A1	A2	HWE	MAF	CALL RATE
3	ADIPOQ	rs1501299	188053816	A	C	0.6378	0.2711	0.92574
3	TFRC	rs2284890	197272543	A	G	0.5831	0.3891	0.8881
3	TFRC	rs41301381	197282716		C	N.A	0	0.97183
3	TFRC	rs3817672	197285207	G	A	0.307	0.4578	0.97183
3	TFRC	rs9877119	197286762			N.A	N.A	0
4	ALB	rs3775485	74494684	T	A	0.05969	0.4369	0.93342
4	ALB	rs962004	74504102	C	T	0.1678	0.4476	0.8924
4	IL8	rs4073	74824887	A	T	0.8194	0.4949	0.8822
4	NFKB1	rs3774932	103643222	A	G	0.5549	0.4347	0.97055
4	NFKB1	rs1801	103720091	C	G	0.5371	0.3892	0.9475
4	UCP1	rs12502572	141704583	A	G	0.8652	0.3138	0.95903
4	UCP1	rs1800592	141713410	G	A	0.4635	0.271	0.97567
4	TLR2	rs4696480	154826575	T	A	0.1236	0.4786	0.95519
4	TLR2	rs3804099	154844105	C	T	0.9336	0.4571	0.7465
4	TLR2	rs3804100	154844858	C	T	0.6464	0.08824	0.95775
4	TLR2	rs5743708	154845766	A	G	0.4117	0.02649	0.96671
5	IL13	rs1800925	132020707	T	C	0.5467	0.1867	0.95006
5	IL4	rs2243248	132036542	G	T	0.7719	0.0678	0.98207
5	IL4	rs2070874	132037608	T	C	0.6698	0.1526	0.95262
5	CD14	rs2569190	139993099	A	G	0.9423	0.4836	0.97695
5	GCCR	rs6195	142759509	G	A	0.5401	0.032	0.96031
5	NR3C1	rs11749561	142771869	T	C	0.76	0.4768	0.8822
5	ADRB2	rs1042711	148186540			N.A	N.A	0
5	ADRB2	rs1042713	148186632	A	G	0.26	0.3641	0.92318
5	ADRB2	rs1042714	148186665	G	C	0.7097	0.4326	0.95903
5	ADRB2	rs1042717	148186838	A	G	0.08141	0.2163	0.5327
5	ADRB2	rs1800888	148187077	T	C	1	0.004781	0.93726
5	ADRB2	rs1042719	148187639	C	G	0.2349	0.3089	0.717
5	IL12B	rs1368439	158674591	G	T	0.8041	0.1771	0.97951
6	LTA	rs909253	31648292	C	T	0.3205	0.3796	0.98335
6	TNF	rs1799964	31650287	C	T	0.1053	0.2017	0.92702
6	TNF	rs1800629	31651010	A	G	0.4232	0.2021	0.9347
6	LY6G5B	rs2142234	31747108	T	C	0.3769	0.06755	0.96671
6	LY6G5B	rs9267532	31747958	T	C	0.5171	0.06166	0.95519
6	LY6G5B	rs1266076	31748497	C	A	0.816	0.3878	0.9411
6	HSPA1L	rs2227956	31886251			N.A	N.A	0
6	HSPA1B	rs6457452	31903529	T	C	0.2676	0.07258	0.95262
6	AGER	rs2070600	32259421	A	G	0.2616	0.07105	0.97311
6	CNR1	rs1049353	88910353	A	G	1	0.2493	0.96799
6	FOXO3	rs9486902	108984744	T	C	0.7922	0.1643	0.98207
6	SGK1	rs1743966	134535639	C	T	0.7239	0.1982	0.8479
6	IFNGR1	rs9389484	137571619			N.A	N.A	0
6	IFNGR1	rs7749390	137582062	G	A	0.5432	0.4199	0.9347
7	IL6	rs1800795	22733169	C	G	0.009327	0.4203	0.97951
7	IL6	rs2069835	22734395	C	T	0.4716	0.05556	0.8204
7	IL6	rs1554606	22735231	T	G	0.07892	0.4463	0.97695
7	IL6	rs2069845	22736673	G	A	0.1884	0.4566	0.97439
7	CAMK2B	rs917791	44235309	C	T	1	0.3681	0.92702
7	CAMK2B	rs10441113	44255189	A	G	0.4365	0.3724	0.96799
7	CAMK2B	rs4526269	44262831	T	G	0.8715	0.3745	0.8924
7	IGFBP3	rs2453839	45920097	C	T	0.6509	0.2012	0.97055
7	IGFBP3	rs3110697	45921554			N.A	N.A	0
7	LEP	rs7799039	127666018	A	G	0.8824	0.4522	0.95134
8	LPL	rs1800590	19840950	G	T	0.429	0.02726	0.96287
8	LPL	rs326	19863718	G	A	0.4711	0.2804	0.96799
8	LPL	rs328	19864003	G	C	0.2588	0.08921	0.96159

CHR	GENE	SNP	BP	A1	A2	HWE	MAF	CALL RATE
8	ADRB3	rs4994	37942954	C	T	0.3894	0.05857	0.5903
8	LY96	rs6472812	75079637	A	G	0.05192	0.04408	0.8932
9	IFNA2	rs624704	21373897	C	T	0.2513	0.2876	0.5787
9	IFNA2	rs632941	21378711	A	G	0.576	0.2768	0.8366
9	DCTN3	rs3802427	34608640	A	G	0.1939	0.1673	0.97951
9	TLR4	rs4986790	119515122	G	A	0.7894	0.07238	0.96415
9	ZER1	rs13284665	130553190			N.A	N.A	0
9	ZER1	rs8507	130555367	G	T	0.2095	0.4543	0.8866
9	ZER1	rs4836625	130556658	T	C	0.1756	0.4972	0.8953
10	MBL2	rs1800450	54201240	A	G	0.000873	0.1731	0.7618
10	MBL2	rs5030737	54201247	T	C	1.44×10^{-7}	0.08491	0.8822
10	MBL2	rs7096206	54201690	G	C	1	0.2023	0.94622
10	TTC18	rs4294502	74705262	C	T	0.4232	0.07464	0.7993
10	TTC18	rs3812621	74706864	G	A	0.5045	0.05785	0.96287
10	ADRB1	rs1801253	115795045	G	C	0.5678	0.2709	0.8853
11	IGF2	rs680	2110209	A	G	0.6978	0.2534	0.95006
11	NUP160	rs11039426	47819694	A	G	0.6922	0.361	0.8677
11	ACTN3	rs1815739	66084670	T	C	0.2458	0.4545	0.98592
11	UCP2	rs660339	73366751	T	C	0.7049	0.4025	0.96543
11	UCP2	rs659366	73372401	T	C	0.1699	0.3591	0.92702
11	UCP3	rs1800849	73397812	T	C	0.9202	0.2413	0.95262
11	IL18	rs360729	111522130	A	T	0.7868	0.2846	0.94494
11	IL18	rs549908	111526125	G	T	0.7811	0.2792	0.9219
11	IL18	rs5744256	111528057	C	T	0.7784	0.2702	0.93598
11	IL18	rs2043055	111536833	G	A	0.3444	0.3637	0.96287
11	IL18	rs187238	111540197	C	G	1	0.2473	0.93982
11	IL18	rs1946519	111540716	A	C	0.6978	0.392	0.93086
11	HYLS1	rs3088241	125268955	C	G	0.1459	0.498	0.96927
11	HYLS1	rs549990	125271253	C	A	0.4411	0.3029	0.7808
11	HYLS1	rs622756	125271382	A	C	0.5832	0.1121	0.97055
11	HYLS1	rs547232	125278208	T	C	0.3577	0.3854	0.97183
12	ADIPOR2	rs16928751	1760459	A	G	0.6067	0.1226	0.92958
12	ADIPOR2	rs35854772	1763337	T	G	0.6073	0.1241	0.9283
12	TNFRSF1A	rs767455	6321205	C	T	0.4898	0.4145	0.92061
12	TNFRSF1A	rs4149570	6321850	T	G	0.4151	0.4149	0.98592
12	GNB3	rs5443	6825135	T	C	0.4493	0.3195	0.8578
12	VDR	rs1544410	46526101	A	G	0.9384	0.4076	0.8843
12	DCD	rs2029851	53327703	G	A	0.2179	0.3032	0.92702
12	IFNG	rs2193049	66833188	C	G	0.7903	0.2849	0.98207
12	IFNG	rs2069727	66834489	G	A	0.5075	0.4697	0.95134
12	IFNG	rs2430561	66838786	A	T	0.6637	0.4751	0.97695
12	IFNG	rs2069709	66839969		G	N.A	0	0.92958
12	IGF1	rs11111272	101351570	G	C	0.09348	0.3019	0.92446
12	IGF1	rs10735380	101368365	G	A	0.7759	0.2767	0.8886
13	FOXO1	rs2701896	40025891	C	G	0.7005	0.3836	0.96799
13	FOXO1	rs17446593	40026084	G	A	0.9043	0.1854	0.97055
13	FARP1	rs3848017	97828589	C	T	0.709	0.2631	0.97823
13	FARP1	rs584800	97836087	A	G	0.6988	0.1715	0.9283
14	NFKBIA	rs696	34940843	A	G	0.5173	0.3546	0.93342
14	AKT1	rs11555433	104312524			N.A	N.A	0
16	TSC2	rs7187438	2060402	C	T	0.9362	0.3461	0.96927
16	LITAF	rs4280262	11554992	G	A	0.3061	0.2322	0.95391
16	MT2A	rs34326929	55199886			N.A	N.A	0
16	MT2A	rs10636	55200843	C	G	0.2132	0.2533	0.97567
16	MT1B	rs1875233	55244204	G	A	0.2336	0.4814	0.92702
16	FOXC2	rs34221221	85157930	C	T	0.03428	0.3867	0.92702

CHR	GENE	SNP	BP	A1	A2	HWE	MAF	CALL RATE
17	CCL2	rs1024611	29603900	C	T	0.1716	0.277	0.8591
17	CCL5	rs2107538	31231892	T	C	1	0.1758	0.8848
17	ACE	rs4295	58910029	G	C	0.2541	0.3933	0.97183
17	ACE	rs4329	58917189	G	A	0.0635	0.4611	0.93726
17	ACE	rs4341	58919721	C	G	0.1141	0.4677	0.8104
17	ACE	rs4362	58927492	C	T	0.603	0.4719	0.93342
17	GPS1	rs4969484	77608149			N.A	N.A	0
18	LPIN2	rs3745012	2910287	T	C	0.5799	0.2743	0.95006
18	APCDD1	rs3748415	10461731	T	C	0.8736	0.1304	0.97695
18	MC4R	rs52820871	56189811	G	T	0.1198	0.01326	0.96543
18	MC4R	rs2229616	56190255	A	G	0.08036	0.01105	0.92702
19	RETN	rs34124816	7639675	C	A	0.6334	0.04128	0.97695
19	RETN	rs1862513	7639792	C	G	0.2029	0.315	0.95519
19	P2RY11	rs12460842	10083194	A	G	4.06×10^{-7}	0.4319	0.8553
19	EIF3G	rs3826785	10088148	T	C	0.8712	0.1304	0.9475
19	ICAM1	rs281432	10251657	G	C	0.4254	0.4681	0.98335
19	ICAM1	rs5498	10256682			N.A	N.A	0
19	GCDH	rs11085824	12862546	G	A	0.5807	0.3601	0.96543
19	GCDH	rs9384	12871642	T	G	0.5857	0.3735	0.96159
19	GDF15	rs1058587	18360421	C	G	1	0.2449	0.94366
19	TGFB1	rs1800469	46552135	T	C	0.8061	0.3218	0.1114
20	TH1L	rs163781	56997159	G	A	0.3757	0.4305	0.97695
20	CTSZ	rs163792	57009741	A	C	0.7675	0.4235	0.97951
22	MIF	rs755622	22566391	G	C	0.1556	0.1919	0.9475
22	PPARA	rs1800206	44992937	G	C	0.5225	0.06345	0.9283

CHR: Chromosome number; BP: SNP base-pair position; A1: Minor allele; A2: Major allele; HWE: Hardy-Weinberg Equilibrium; MAF: minor allele frequency (from main study population)
Lines in red denote SNPs removed from final analysis.

The general characteristics of the study population are presented in Table 6.3. Average age of the patient cohort at diagnosis was 65.5 ± 11.8 years (mean ± S.D). The majority of patients were diagnosed with stage III or IV cancers. Average weight loss was 6.9 ± 9.8% with a mean BMI of 24.9 ± 4.9 at diagnosis. Of the patients in whom CRP levels were assessed (n=569), 58.7% had a CRP concentration of >10 mg/l. There were no significant differences in age, stage of disease, BMI and percentage weight loss between patients with CRP measured and the entire cohort.

Table 6.3 Patient demographics (main cohort)

Patients recruited from (2004 – 2008): NHS Lothian, UK; Cross Cancer Institute, Edmonton, Canada; and McGill University Health Centre, Montreal, Canada.

	No. of patients (n=775)
Age (years) †	65.5 ± 11.8
Range	(27 -97)
Sex	
M	476 (61.4)
F	299 (38.6)
Tumour type	
Oesophageal or Gastric	389 (50.2)
Pancreatic	114 (14.7)
Non-small cell lung cancer	232 (29.9)
Other	40 (5.2)
Stage	
I	38 (4.9)
II	95 (12.3)
III	216 (27.9)
IV	392 (50.5)
Unknown	34 (4.4)
Body mass index (kg/m^2) †	24.9 ± 4.9
Range	(12.9 – 46.7)
Percentage weight loss ‡	7.95 ± 8.16
Range	(0 – 43.8)
C-reactive Protein (mg/l)† (n=569)	23.0 ± 35.9
CRP > 10 mg/l	235 (41.3)
CRP ≤ 10 mg/l	334 (58.7)

Values are number of patients with percentages in parentheses unless indicated otherwise; †values are mean ± SD. Characteristic s were measured at first presentation to a surgical or oncology clinic.

Table 6.4 lists the detailed results for SNPs with permutated *p* values of <0.05 for each cachexia phenotype. Eight SNPs have associations of *p* <0.02 with various cachexia phenotypes. Three of these SNPs are found within chromosome 1 in the genes *SELP, LEPR and DIO1;* three within chromosome 3 in the genes *APEH and GHRL*, one within chromosome 12 in the *TNFRSF1A* gene, and one within chromosome 19 in the *ICAM1* gene. SNPs found on the same chromosomal region (within 10,000kb) were grouped together to form haplotypes. The haplotypes formed by the rs4855881 and rs2960548 SNPs in the *APEH* gene failed to show any significant association with weight loss.

Table 6.4a Genes with variants significantly associated with cancer cachexia in patients classified according to weight loss alone

Weight loss>15%. Number affected: *145/775 (18.7%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
SELP	rs6136	C	0.31 (0.14 – 0.72)	0.006615	0.008062
ICAM1	rs281432	G	1.53 (1.06 – 2.20)	0.02163	0.01652
DIO1	rs11206244	T	1.54 (1.06 – 2.24)	0.0226	0.02164
ADIPOR2	rs16928751	A	0.53 (0.29 – 0.96)	0.03521	0.03053
APEH	rs2960548	G	1.48 (1.03 – 2.11)	0.03384	0.03768
Weight loss>10%. *Number affected: 266/775 (34.3%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
LEPR	rs1137100	G	0.66 (0.47 – 0.92)	0.01494	0.013
DIO1	rs11206244	T	1.52 (1.09 – 2.11)	0.0129	0.01512
SELP	rs6136	C	0.52 (0.29 – 0.93)	0.02746	0.02581
HYLS1	rs3088241	C	0.72 (0.53 – 0.97)	0.02829	0.02709
CAMK2B	rs10441113	A	0.73 (0.54 – 0.99)	0.04096	0.03419
Weight loss>5%. *Number affected: 415/775 (53.5%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
TNFRSF1A	rs4149570	T	1.42 (1.08 – 1.87)	0.01134	0.01759
TNFRSF1A	rs767455	C	0.71 (0.53 – 0.95)	0.02034	0.02275
TNFRSF1B	rs976881	A	0.76 (0.57 – 1.00)	0.04804	0.04324
IL18	rs1946519	A	1.35 (1.02 – 1.79)	0.03895	0.04969

Table 6.4b *Genes with variants significantly associated with cancer cachexia in patients classified according to weight loss with systemic inflammation (CRP >10mg/l)*

Weight loss>15% & CRP>10mg/l. *Number affected: 76/569 (13.4%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
APEH	rs2960548	G	2.17 (1.36 – 3.47)	0.001125	0.000997
GHRL	rs42451	T	2.04 (1.25 – 3.31)	0.004031	0.004058
TNFRSF1A	rs4149570	T	1.84 (1.16 – 2.92)	0.009322	0.01031
SELP	rs6136	C	0.26 (0.08 – 0.79)	0.01765	0.01103
CNR1	rs1049353	A	1.82 (1.08 – 3.06)	0.02366	0.02254
IRS1	rs1025333	A	2.24 (1.07 – 4.69)	0.03257	0.03183
APEH	rs4855881	C	1.64 (1.04 – 2.59)	0.03431	0.03191
FOXO1	rs17446593	G	0.49 (0.26 – 0.92)	0.02704	0.03239
ICAM1	rs281432	G	1.63 (1.04 – 2.54)	0.03276	0.03941
Weight loss>10% & CRP>10mg/l. *Number affected: 123/569 (21.6%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
APEH	rs2960548	G	1.80 (1.21 – 2.68)	0.003528	0.003499
GHRL	rs42451	T	1.79 (1.18 – 2.72)	0.006219	0.00467
TNFRSF1A	rs4149570	T	1.51 (1.04 – 2.18)	0.02958	0.01998
HYLS1	rs3088241	C	0.66 (0.46 – 0.95)	0.02374	0.02074
APEH	rs4855881	C	1.57 (1.06 – 2.32)	0.02334	0.02847
TSC2	rs7187438	C	0.64 (0.43 – 0.95)	0.0265	0.03438
TNFRSF1B	rs3397	C	0.67 (0.46 – 0.97)	0.03527	0.04286
Weight loss>5% & CRP>10mg/l. *Number affected: 166/569 (29.2%)*					
Gene	SNP	Risk allele	OR (95% C.I)	*p* Value	Permutated *p*
APEH	rs2960548	G	1.67 (1.17 – 2.38)	0.004924	0.004533
APEH	rs4855881	C	1.56 (1.10 – 2.21)	0.01321	0.01212
TNFRSF1A	rs4149570	T	1.51 (1.08 – 2.10)	0.01559	0.02074
ADIPOR2	rs16928751	A	0.56 (0.33 – 0.95)	0.03308	0.02096
ADIPOR2	rs35854772	T	0.57 (0.33 – 0.97)	0.03733	0.02667
TNFRSF1B	rs3397	C	0.70 (0.50 – 0.98)	0.03944	0.02923
LTBP1	rs817529	G	0.70 (0.49 – 0.98)	0.03719	0.03791
TNFRSF1A	rs767455	C	0.68 (0.48 – 0.96)	0.02682	0.03846

Analyses of candidate gene groups based on functional similarity revealed three groups that were associated with at least one cachexia phenotype at the $p<0.05$ level (Table 6.5).

Table 6.5 Candidate gene groups associated with cancer cachexia phenotypes

Phenotype	Candidate gene group function	Number of genes[†]	Number of SNPs	*p* values
Weight loss >10% & CRP >10 mg/l	Appetite regulation	2	3	0.0155
	Glucocorticoid signalling	4	9	0.0351
	MAPK activity regulation	7	14	0.0481
Weight loss >15% & CRP >10 mg/l	Appetite regulation	2	3	0.008499
	Glucocorticoid signalling	4	9	0.0181
	MAPK activity regulation	7	14	0.0264

[†] The genes in each candidate gene group are listed in supplementary table 6.1.

Validation study

A total of 101 patients were recruited independently for the validation study. Patient demographics of the validation cohort are presented in Table 6.6. Study subjects were genotyped for SNPs with $p<0.05$ in the main study.

Table 6.6 Patient demographics (validation cohort)

Patients recruited from (2007 – 2008): Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland.

	No. of patients (n=101)
Age (years) †	62.0 ± 11.5
Range	(35 – 88)
Sex	
M	60 (59.4)
F	41 (40.6)
Tumour type	
Oesophageal or Gastric	18 (17.8)
Pancreatic	6 (5.9)
Non-small cell lung cancer	19 (18.8)
Other	58 (57.4)
Stage	
I	0
II	3 (3.0)
III	2 (2.0)
IV	96 (95.0)
Body mass index (kg/m^2) †	23.7 ± 4.3
Range	(15.4 – 37.8)
Percentage weight loss †	5.54 ± 7.91
Range	(0 – 43.1)
C-reactive Protein (mg/l)† (n=95)	75.5 ± 76.4
CRP > 10 mg/l	78 (77.2)
CRP ≤ 10 mg/l	17 (16.8)

Values are number of patients with percentages in parentheses unless indicated otherwise; †values are mean ± SD. Characteristic s were measured at first presentation to a oncology clinic.

One replication of the main study was found. The C allele of the rs6136 SNP was inversely associated with weight loss >10% in the main study (OR 0.52, 95% C.I 0.29 – 0.93, p =

0.026) as well as in the validation study (OR 0.09, 95% C.I 0.01 – 0.98, $p = 0.035$). However, there was no association with weight loss >15% (OR 0.31, 95% C.I 0.03 – 3.60, $p = 0.53$)

6.5 Discussion

This study has identified that individuals who carry the C-allele of the rs6136 polymorphism are at reduced risk of developing cachexia as defined by weight loss >10%. The *SELP* gene which encodes P-selectin is a candidate for the development of cachexia due to its role in the recruitment of neutrophils and macrophages in the inflammatory response. The C allele of the non-synonymous intronic variant, rs6136 has been previously associated with decreased serum P-selectin levels (Miller *et al*, 2004; Volcik *et al*, 2006).

In cachexia, as with a study of with many potential polygenic traits, one of the main obstacles is the lack of a uniform phenotype. However, a recent consensus document on the definition of cachexia suggested a key component of cachexia to be at least a 5% loss of oedema-free body weight during the previous 12 months or less (Evans *et al*, 2008). The document also suggested classifying the degree of cachexia as mild, moderate or severe, depending on whether the observed weight loss within the previous 12 months (or less) was >5%, >10% or >15%, respectively. Hence in the present study we considered cachexia as a spectrum using these cut-offs.

CRP has been studied in a wide variety of tumour types and has been linked to poorer survival (Mahmoud & Rivera, 2002; McMillan *et al*, 2003). CRP has also been incorporated into a three-factor mathematical definition of cachexia for patients with pancreatic cancer (Fearon *et al*, 2006). The multi-profile definition was found to have more prognostic value compared with weight loss alone. To reflect that cachexia represents a spectrum and that

the presence of systemic inflammation with weight loss may represent a unique sub-phenotype of cachexia which confers an increased mortality risk, we have chosen to study cachexia across three different percentage weight loss categories alone and with the presence of an increased CRP concentration in comparison with a weight-stable phenotype (i.e. ≤5% weight loss). Clearly, much work is required before fully validated definitions of cachexia are available. Until then, it appears reasonable to investigate cachexia based on the present arbitrary definitions.

Patients in the present study were recruited at various stages of the disease process. We have attempted to address this issue by adjusting the analyses for tumour stage at the time of recruitment. The amount of weight lost during the cancer journey may be affected by patients' pre-morbid BMI. The initially overweight/obese cancer patient may be more likely to lose a greater amount weight compared with a patient with the same cancer type in the normal BMI range over the same period of time. To account for this variation, the analyses have also been adjusted for pre-morbid BMI.

The present study represents the first large scale candidate gene association study of cancer cachexia spanning a wide variety of genes such as genes that regulate inflammation, muscle and adipose tissue metabolism and appetite. SNPs chosen for the study were based on a literature review of SNPs with known functional effects and/or clinical relevance with regard to the development of cachexia. SNPs based on 18 genes identified in a gene expression study on muscle samples in patients with cancer cachexia were also analysed. Instead of utilising a tag SNP approach, as it was not realistic to analyze all possible gene variants and combinations, SNPs most likely to be functional (i.e. within exons, non-synomymous and with a MAF of >0.1) and hence more likely to be associated with the development of cachexia were selected.

To further add strength to the study, a separate validation study was done by replicating the association study in an independently recruited group of patients. In the initial exploratory cohort we identified 21 SNPs with significant associations with cachexia phenotypes. However, when both the exploratory and validation cohorts were considered, only cancer patients carrying the minor allele (C) of rs6136 were found to be at reduced risk of developing cachexia as defined by weight loss >10% (main study (OR 0.52, 95% C.I 0.29 – 0.93, p = 0.026); validation study (OR 0.09, 95% C.I 0.01 – 0.98, p = 0.035)). Unfortunately, the validation study failed to confirm other significant associations found from the main cohort. This may be due to the small sample size of the validation study which is a key limitation. In addition, due to the small sample size of the validation cohort, gene group analysis was only performed on the main cohort.

The gene group analysis performed provides one way of summarising the evidence between cachexia traits and multiple genetic variants across groups of genes that share functional similarity. Appetite regulation was found to be most significantly associated with the cachexia trait weight loss >15% and CRP >10 mg/l (p = 0.008). There has been some evidence to date that negative regulators of appetite are elevated in cachexia (Doehner *et al*, 2001; le Roux *et al*, 2005). A number of animal studies have also shown prevention or reversal of cachexia by deletion or blockade of specific appetite pathways (Marks *et al*, 2001; Nicholson *et al*, 2006; Wisse *et al*, 2001).

In addition to the above link, the glucocorticoid signalling pathway was also found to be associated with cachexia (weight loss >15% and CRP >10 mg/l) (p = 0.0181). There is evidence that glucocorticoids and its associated signalling pathway are involved in accelerating protein degradation in muscle, which results in loss of lean body mass in cachexia (Tisdale, 2009). Glucocorticoids work thorough a permissive effect on the upregulation of messenger RNA and the subsequent synthesis of components of the ubiquitin–proteasome system in muscle. Glucocorticoids inhibit protein synthesis and

promote gluconeogenesis, and suppress glucose and amino acid muscle uptake by inhibiting cellular transporters (Lecker *et al*, 2006). Mitogen activated protein kinases (MAPK) activity regulation was also found to be associated with cachexia (weight loss >15% and CRP >10 mg/l) (p = 0.0264). MAPKs are known to mediate lipolysis in cancer cachexia (Ryden & Arner, 2007), and are also potential regulators of muscle catabolism in cachexia (Keren *et al*, 2006).

Previous genetic studies on cancer cachexia have identified associations with cachexia and polymorphisms in cytokine genes such as the *IL1B* 3954C/T polymorphism (rs1143634) in patients with gastric cancer (Zhang *et al*, 2007), and the *IL10* -1082A/G polymorphism (rs1800896) in patients with gastro-oesophageal cancer (Deans *et al*, 2009a). Cancer related anorexia has been associated with the *TNF* -308G/A polymorphism (rs1800629) in patients with non-small cell lung cancer (Jatoi *et al*, 2009). Despite some significant associations with other polymorphisms in pro-inflammatory cytokines genes (Table 2), the present study was unable to confirm the previous specific associations in the present study. However, all these studies have focused only on one particular type of cancer and on a small number of genetic variants. More widely applicable biomarkers may prove more useful. One of the strengths of the present study is the analysis of a wide variety of candidate genes that may influence the development of cachexia in patients with various cancer types.

The nature of cancer cachexia dictates that there are fewer individuals who develop the most severe aspects of the syndrome. At the severe end of the cachexia spectrum, the power in the present study to detect weak associations with uncommon variants was low. It may be that a larger sample size may be required to fully elucidate the effects of such variants in individuals with severe or refractory cachexia.

The diverse cachexia phenotypes we investigated represent various stages in the cachexia journey with potential genetic influences at each stage. The present chapter suggests that

multiple pathways are likely to be involved in the pathogenesis of cancer cachexia and, in particular, appetite regulation, glucocorticoid signalling, and MAPK activity regulation may have central roles in this process and should be further investigated. Individuals with the C-allele of the rs6136 P-selectin polymorphism appear to be at reduced risk of developing cachexia.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE WORK

Cancer cachexia significantly impairs quality of life and response to treatment and is associated with increasing morbidity and mortality. This thesis set out to investigate the time course changes in regional body fat and lean tissue compartments in the cachexia journey using pancreatic cancer as a model for cachexia. The prognostic value of body composition, specifically skeletal muscle was also evaluated. The thesis also sought to determine whether there is a genetic predisposition to cancer cachexia.

7.1 Evaluation of body composition in patients with pancreatic cancer as a model of cachexia

Patients with pancreatic cancer have long been associated with the most severe forms of cachexia. Recent studies have revealed that a substantial proportion of cancer patients at diagnosis now have a BMI in the overweight range (Irigaray *et al*, 2007). Chapter 3 showed a similar finding: patients had a mean BMI of 23.9 with 40% of individuals being in the overweight/obese range. However, underneath this mantle of adipose tissue the previously noted tendency to muscle wasting was observed. The use of CT images in the cross-sectional component of the study identified that 56% of patients had sarcopenia at the time of presentation. Moreover, in the longitudinal part of the study, the tendency to muscle loss continued in at least a proportion of patients.

It was interesting, however, that in the longitudinal study a much greater rate of fat loss was observed as compared with muscle loss. One hypothesis would be that during cancer cachexia, muscle loss is an early feature but that subsequently at least a proportion of patients are able to activate compensatory mechanisms aimed at conserving muscle. It is

also possible that the degree of metabolic change versus reduced food intake (simple starvation) may vary according to the patients' clinical status and concomitant anti-cancer therapy. Another possibility is that in at least a proportion of patients there is a genetic predisposition or resistance to cachexia and that this genotype may relate to phenotype during different phases of the cancer/cachexia journey. There is increasing evidence that gene polymorphisms are related to cancer cachexia susceptibility (Deans *et al*, 2009a; Vigano *et al*, 2009).

Overall, the longitudinal study shows that both muscle and fat loss are predominant features in cancer cachexia. This suggests that any future definitions of cancer cachexia should include the loss of both muscle and fat and not just muscle alone.

The longitudinal study in Chapter 3 reveals that the loss of fat mass was 40,840 kcal over 135 days which equates to a negative energy balance of approximately 300 kcal/day. These findings are consistent with previous studies, which have demonstrated that the daily energy deficit in advanced cancer patients with weight loss is about 300kcal/d (Moses *et al*, 2004). This energy deficit is due to a combination of reduced food intake and increased energy expenditure (Wigmore *et al*, 1997b).

The use of 1 kcal/mL oral nutritional supplements has not been shown to improve the nutritional status of patients on chemotherapy (Ovesen *et al*, 1993). However, recent studies using a more calorie dense (1.5 kcals/mL) and higher protein supplementation have suggested that at least weight stabilization can be achieved in some patients (Kumar *et al*, 2010). Whist patients' energy deficit may be addressed by such nutritional support, it is evident that attenuation of loss of lean tissue/muscle mass is more difficult to achieve (Nixon *et al*, 1981). This suggests that in addition to calorie supplementation, other treatment options are required to reverse/attenuate the loss of lean body mass such as improving anabolism and/or treating hypercatabolism. One of the first steps in designing intervention

trials to address this issue would be to have an accurate measure of lean or skeletal muscle mass.

A BMI <18.5kg/m^2 is considered by many authorities to represent an individual at serious risk of undernutrition (Shetty, 2003). The study in Chapter 3 showed only 10% of individuals at baseline fulfilled this criterion. Given the prevalence of overweight/obesity (40%) it would seem unlikely that even in the presence of ongoing weight loss, the majority would reach this boundary (i.e. a BMI <18.5kg/m^2) at or near the time of death. However, BMI has clear limitations and a more detailed evaluation of body composition in patients with advanced pancreatic cancer revealed significant wasting of the lean tissues, with a majority of patients below or well below benchmark levels of muscularity known to be associated with mortality and functional disability (Janssen *et al*, 2002). Current literature reveals that concurrent low muscle mass (sarcopenia) but a high fat mass is a worst case scenario (Bigaard *et al*, 2004; Heitmann *et al*, 2000; Honda *et al*, 2007; Prado *et al*, 2008), and this is clearly apparent in the study group, where sarcopenic overweight/obese patients had the worst prognosis overall, even compared with patients who were sarcopenic and had a BMI in the normal or underweight range.

The mechanism that links sarcopenic overweight/obesity with accelerated demise is not known. However, muscle wasting is a known complication associated with insulin resistance found commonly in obesity. Insulin resistance causes muscle wasting by mechanisms that involve suppression of PI3K/Akt signaling leading to activation of caspase-3 and the ubiquitin-proteasome proteolytic pathway causing muscle protein degradation.(Wang *et al*, 2006).

A weakness of the study in Chapter 3 is that the population assessed in the longitudinal study is quite small (n=44) and there were no details of treatment and response available to assess the effects of the latter on changes in body composition. Nevertheless, Chapter 3 has shown that the presence of overweight/obese sarcopenia in pancreatic cancer patients is an adverse prognostic factor. This finding mirrors a previous study on a cohort of lung cancer patients (n = 250) undergoing chemotherapy (Prado *et al*, 2008).

One important issue is that in any intervention trial in cancer patients with sarcopenic obesity, the emphasis must be on increasing levels of muscularity whilst avoiding making the obesity worse. Thus the use of simple oral nutritional supplements would need to be combined with exercise and other promoters of net muscle anabolism. The use of serial body composition analysis derived from diagnostic CTs would be one way of monitoring the success of such a regimen.

7.1.1 General application of CT scanning for body composition analysis

CT is capable of distinguishing between different tissue types on the basis of their attenuation characteristics (Hounsfield units). Regional analysis of fat and lean tissue (e.g. L3 level) are highly correlated to corresponding whole body compartments and can provide precise quantification of specific adipose tissues, skeletal muscles and organs (Heymsfield *et al*, 1997). CT is currently considered a gold standard imaging method of body composition analysis at the tissue-organ level (Prado *et al*, 2009b).

The use of CT analysis to investigate body composition and body composition changes throughout the disease trajectory represents an optimal choice because of the ability to discriminate individual tissues and the number of scans available over time for longitudinal

studies. Within-scanner reproducibility and between-scanner agreement have been shown to be high for measurements of adipose and muscle tissue area by CT although caveat must be attached to a standard error (SE) of ~1.1% for repeated measures of muscle tissues and ~5.5% for adipose tissues (Brandberg *et al.* unpublished work).

A problem with multiple CT images over the whole body is the high radiation absorbed dose, resulting in effective doses of the order of 10 mSv per investigation (Mattsson & Thomas, 2006). This limitation is accepted for patients with cancer because of the ability to follow tumour growth and response to therapy. Cancer patients are routinely evaluated by high-resolution diagnostic imaging and Chapter 3 has shown that routine diagnostic CT scans are a good resource for detailed nutrition/metabolic assessment of patients and the identification of overweight/ obese sarcopenia.

One possible issue with CT image-based body composition analysis is that while this is highly robust in terms of delineating and quantifying muscle and adipose tissue, there have been few studies on the usefulness of CT images on assessing quality of tissue assessed, in particular skeletal muscle. Thus, at the most basic level, although the volume of a muscle may be delineated, the protein content remains unknown and the assumption that this remains constant is untested.

The recent consensus article on cachexia (Evans *et al*, 2008) recognises the key importance of skeletal muscle wasting as a signature event in cancer cachexia. Chapter 3 has demonstrated that routine diagnostic CT scans can be used to derive an index of muscle mass. With such methodology it is possible to classify patients as sarcopenic or not. Sarcopenia has been linked to poorer prognosis in obese/overweight cancer patients. Recent studies have also shown that cancer patients with sarcopenia may be prone to severe toxicity during systemic therapy (Prado *et al*, 2007; Prado *et al*, 2009a). There is a strong argument for all cancer patients to have their diagnostic CT scans analysed for body

composition in terms of stratifying cancer patients' entering cachexia clinical trials, for dose adjustment of chemotherapy to avoid systemic complications or to guide optimal nutritional and metabolic care within support care programs.

7.2 Identification of candidate genes for cancer cachexia (a systematic literature review)

The finding that only a proportion of patients with chronic disease develop cachexia has prompted studies looking for genetic polymorphisms that may underlie differential susceptibility (Tan *et al*, 2008). Based on the hypothesis that continued systemic inflammation plays a central role to the pathogenesis of cachexia, the most frequent targets for these studies have been genes encoding pro-inflammatory cytokines. While much attention has been placed on cytokine polymorphisms and their association with cachexia, it is of note that mechanisms surrounding the pathogenesis of cachexia is complex and is likely to involve gene products which act both upstream and downstream of cytokines. Pathways not affected by cytokines may also play a role in the development of cachexia. Chapter 4 investigates by means of a systematic literature review all genetic polymorphisms with known functional or clinical significance in potential candidate genes involved in the development of cancer cachexia. Domains investigated include systemic inflammation, central energy balance, control of muscle metabolism/function, control of adipose tissue metabolism/function, and regulation of appetite all of which have been implicated in the pathogenesis of cancer cachexia.

Like many complex conditions and diseases, the risk of developing cancer cachexia is probably determined by multiple genetic factors and environmental factors are likely to add to the heterogeneity of the condition. Although the number of reports on polymorphic gene

variants associated with multi-factorial diseases and conditions are growing dramatically, very few studies provide firm and reliable evidence of causative relationships between these polymorphisms and risk or pathogenesis. This could be due to issues concerning phenotype misclassification (which is particularly relevant to cancer cachexia as the agreed definitions of the syndrome are arbitrary and await validation), epistatic interaction and undetected genomic heterogeneity in the underlying population (Tan *et al*, 2008). Of note, in a review of allelic association with common disease phenotypes, only six of 166 associations subjected to multiple evaluations were confirmed consistently (Hirschhorn *et al*, 2002). Possible causes of false-positive association studies include population stratification, variable linkage disequilibrium and genotype misclassification. In addition, in many of these studies, the possible effects of single gene variants were assessed in situations when combined impacts of multiple factors could be expected (Loktionov, 2003). In chapter 4, a total of 184 polymorphisms with functional and/or clinical significance in terms of cachexia were identified in 92 genes. However, only 42 polymorphisms have been identified with a potential role in the development of cachexia and have been independently verified in at least one repeat study.

Of the 42 polymorphisms with a potential role in the development of cachexia that have been verified independently in at least one repeat study, 13 polymorphisms have been shown to have more than one effect on clinical features associated with cancer cachexia (i.e. systemic inflammation, loss of muscle/adipose tissue mass, reduced cancer survival). These 13 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers for cancer cachexia.

The analysis of candidate genes relies on an understanding of disease pathophysiology. However, candidate gene studies are limited by the fact that they rely on an assumed

knowledge about the pathogenesis of cancer cachexia. The selection of genes for study is based on an *a priori* hypothesis about disease causality. A good case can usually be made to study many of the 30,000 genes on the human genome so, in the context of a disease that is complex and polygenic, candidate gene analysis based merely on a "scientific hunch" is a rather naive and approach that will be rarely successful. Chapter 4 has therefore provided an excellent initial framework in which to select and study the possible genetic variance associated with developing cancer cachexia.

Since the completion of the literature review, apart from two studies confirming the association of *IL10* and *IL8* polymorphisms with cancer cachexia (Bo *et al*, 2010; Sun *et al*, 2010), there have not been any other significant genetic association studies on cachexia. However, a recent study showed that blockade of the activin type-2 receptor (ActRIIB) in mice results reverses cancer-induced muscle loss and anorexia (Zhou *et al*, 2010). This suggests that the ActRIIB pathway is likely to play an important role in the development of cachexia and variants of genes involved in the pathway should be further investigated for association with cachexia.

Over the past few years, catalysed in part by technological advances allowing cheaper genotyping, and the completion of the HapMap project, genome wide association studies (GWAS) have become increasingly common. This technique is superior to candidate gene studies alone as it allows researchers the advantage of being able to identify genes in a "hypothesis free" manner, rather than identify selecting candidate genes on the basis of likely causality. The genome-wide association approach therefore represents an unbiased yet fairly comprehensive option that can be attempted even in the absence of convincing evidence regarding the function of the genes (Hirschhorn & Daly, 2005). The full publication of the HapMap project data has facilitated genetic mapping studies across a broad array of

complex phenotypes for use in genetic case control studies, and to identify sets of SNPs that take advantage of the LD patterns of the genome and allow more economical genotyping though indirect association studies (2005).

The ability to simultaneously examine hundreds of thousands of variants throughout the genome is both the strength and the weakness of the GWAS approach. The power of GWAS is that they provide a relatively unbiased examination of the entire genome for common risk variants; their weakness is that in doing so, they swamp the signal from true risk variants with statistical noise from the vast numbers of markers that are not associated with disease. To separate true signals from noise, researchers have to set an exceptionally high threshold that a marker needs to exceed before it is accepted as a likely disease-causing candidate. The latter reduces the problem of false positives, but it also means that any true disease markers with small effects are lost in the background noise (Pearson & Manolio, 2008). GWAS association studies are also considerably more expensive compared with candidate gene studies and require much larger sample sizes to generate meaningful data.

7.3 Identification of candidate genes for cancer cachexia (gene expression microarray study)

The maintenance of muscle mass is a balance between protein synthesis and degradation. In cachexia, this balance is disrupted leading to a decrease in protein synthesis or an increase in protein degradation or a combination of both.

Gene expression technology using gene microarrays allows a comprehensive picture of gene expression at the tissue and cellular level, thus helping to understand the underlying

physiological and pathological processes in cancer cachexia. In chapter 5, the differential expression of genes in *rectus abdominis* muscle of cachetic versus non-cachetic patients was studied. The study revealed an 83-gene signature that correlated with percentage weight loss (74 genes correlated positively with weight loss and 9 correlated negatively with weight loss).

A significant weakness of the study was the small sample size. Also, there was a potential issue that patients with cancer and normal controls were analysed together and perhaps studies with proper control groups are necessary. Due to statistical issues raised by microarray technology, it is also essential that findings be confirmed using independent methodological criteria. A follow-up study done using quantitative RT-PCR on nine selected genes from the 83 genes validated the results of eight genes from the microarray analysis while one (*SGK*) was inconsistent with the microarray data (Stephens *et al*, 2010).

Although the Affymetrix genechip array allows study of the differential expression of a large number of genes (approx.38,500 genes), it does not cover all the genes within the human genome. It is therefore likely that some important genes involved in the pathogenesis of cachexia may be missed.

Another issue with microarray studies are that steady-state levels of RNA are not necessarily reflective of the final steady-state level of the functional protein translation product of an mRNA and further studies might be required involving the use of specific antibody probes in Western blot or immunocytochemical studies (Murphy, 2002). Although a microarray experiment might indicate an increase or decrease in the expression of a gene, an independent method might reveal a greater or a lesser change. Does such a result represent

sufficient confirmation of the microarray findings, or does a quantitative difference raise new questions about the validity of the microarray data?

Nevertheless, the study in chapter 5 has shown a variety of genes which are correlated with weight loss and may represent novel biomarkers for cancer cachexia. Polymorphisms in the genes identified in this chapter may play a role in the inter-individual variability of developing cachexia and all genes that were negatively correlated with weight loss and genes with a positive correlation of r >0.65 with weight loss were further studied in the genetic association study (see below)

7.4 Candidate gene association study of cancer cachexia

191 SNPs across 99 genes were selected for the candidate gene association study. SNPs chosen for the study were based on the literature review of SNPs with known functional effects and/or clinical relevance with regard to the development of cachexia (Chapter 3). A decision was also made to analyse SNPs based on 18 genes identified in a gene expression study on muscle wasting in patients with cancer cachexia (Chapter 4). Instead of utilising a tag SNP approach, as it was not realistic to analyse all possible gene variants and combinations due to limitation of resources, SNPs selected were those most likely to be functional (i.e. within exons, non-synonymous and with a MAF of >0.1) and hence more likely to be associated with the development of cachexia. The study was carried out in 775 patients also carried out using polymorphisms significantly associated with cachexia in the main cohort of patients.

7.4.1 The P-selectin (*SELP*) rs6136 polymorphism is associated with the development of cachexia

21 SNPs were found to have significant associations with cachexia in the main study. However, only the C allele (minor allele frequency 10.7%) of the rs6136 (*SELP*) SNP was found to be associated with a reduced risk of developing cachexia (weight loss >10%) both in the main study and the validation study. The C allele of the non-synonymous intronic variant, rs6136 has been previously associated with decreased serum P-selectin levels (Miller *et al*, 2004; Volcik *et al*, 2006).

The role of P-selectin in the genesis of cachexia remains to be determined. However, P-selectin has been characterized previously by approaches such as gene knockout or the use of specific inhibitors to be involved in the recruitment of neutrophils and macrophages in inflammatory responses (Borges *et al*, 1997; Chen & Geng, 2006). P-selectin may participate in intra-tumoural regulation of the genesis of systemic inflammation via the innate immune system and/or regulation of the complex interaction within muscle between the endothelium and signalling pathways in muscle fibres (Wagenmakers *et al*, 2006). The above suggest that P-selectin is likely to play a causal role in the development of cancer cachexia.

The results in Chapter 6 should be interpreted considering several limitations. First, candidate genes in the study were selected based on educated guesses on which polymorphisms are most likely to be associated with cachexia phenotypes. Given unlimited resources, it would have been more informative using a tag SNP approach to investigate all the genes identified in Chapters 4 and 5. Second, the sample size of the main population was relatively small and for the least prevalent cachexia phenotypes, there was insufficient power to detect association with an OR >1.5 especially with polymorphisms with a low MAF. Another key limitation was the small sample size of the validation cohort which may have led to the inability to confirm other significant associations observed in the main study. Future

studies should involve recruiting a larger cohort of patients both of the main and validation cohorts.

Third, there are certain features of the study which may lead to confounding results. Patients recruited were heterogeneous in terms of diagnosis and across different population groups. It could be argued, however, to a certain extent that these results should then be replicable and valid for all types of cancer and across all population groups. Patients were also recruited at various stages of the disease process therefore there may be significant variation in the time frame for weight loss. This was addressed by adjusting for stage of disease assuming that patients who are diagnosed with more advanced disease would present with greater amount of weight loss.

Chapter 6 has shown that individuals with the C-allele of the rs6136 P-selectin polymorphism appear to be at reduced risk of developing cachexia. This information may prove useful in the risk stratification of pre-cachetic cancer patients, although the question may be asked is genotyping for a SNP that is only present in ~11% of the population worthwhile?

7.4.2 Gene groups regulating appetite, glucocorticoid signalling, and mitogen activated protein kinases (MAPK) activity may have central roles in the pathogenesis of cancer cachexia.

Candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology and the association between grouped SNPs and cachexia were analysed using subject in the main study cohort. Gene group analysis performed provides one way of summarising the evidence between cachexia traits and multiple genetic variants across groups of genes that share functional similarity.

Gene groups regulating appetite, glucocorticoid signalling, and mitogen activated protein kinases (MAPK) activity were associated with cachexia (weight loss >15% and CRP >10mg/l).

Previous studies have shown that negative regulators of appetite are elevated in cachexia (Doehner *et al*, 2001; le Roux *et al*, 2005). The study in Chapter 3 has also identified the presence of an energy deficit of approximately 300 kcal/day which may result from reduced food intake. The gene association study further confirms anorexia as an important variable in the development of cachexia.

A number of animal studies have shown prevention or reversal of cachexia by deletion or blockade of specific appetite pathways (Marks *et al*, 2001; Nicholson *et al*, 2006; Wisse *et al*, 2001). Since anorexia is a key component of cancer cachexia, one treatment strategy is to improve appetite by using appetite stimulants to ensure adequate intake of nutrients. These include orexigenic agents such as Megesterol acetate, cannabinoids, and 5-HT3 antagonists. All these appetite stimulants have been shown to ameliorate or slow anorexia and unintentional weight loss in other populations but have not shown promise in increasing lean body mass or markers of protein metabolism in the cachectic cancer patient (Kumar *et al*, 2010).

There is evidence that glucocorticoids and its associated signalling pathway are involved in accelerating protein degradation in muscle, which results in loss of lean body mass in cachexia (Tisdale, 2009). Glucocorticoids work thorough a permissive effect on the up-regulation of messenger RNA and the subsequent synthesis of components of the ubiquitin–proteasome system in muscle. Glucocorticoids also inhibit protein synthesis and promote gluconeogenesis, and suppress glucose and amino acid muscle uptake by inhibiting cellular transporters (Lecker *et al*, 2006). Many pathological conditions characterized by muscle

atrophy such as cachexia are associated with increase in circulating glucocorticoids levels (Lecker *et al*, 1999), suggesting that these hormones could trigger the muscle atrophy observed. The study in Chapter 6 reinforces that the glucocorticoid signalling pathway may play an important role in the development of cachexia.

Experiments with the glucocorticoid receptor antagonist RU-486 in mouse models show poor results and do not ameliorate the detrimental wasting of muscle and adipose tissues seen in a well-characterized murine tumour-induced cachexia model (Rivadeneira *et al*, 1999). Therapies based on other components of the glucocorticoid pathway may, however, prove beneficial and should be investigated further. For example, glucocorticoids can cause muscle atrophy by altering the muscle production of IGF-I and myostatin, two growth factors exhibiting opposite effects on muscle mass development (Schakman *et al*, 2008). Therefore, IGF-I stimulation or myostatin blockade constitutes some of the most promising future therapeutic approaches to prevent muscle atrophy caused by glucocorticoids.

Pathways regulated by pro-inflammatory cytokines include those activated by the MAPKs: ERK, JNK, and p38. MAPKs are known to mediate lipolysis in cancer cachexia (Ryden & Arner, 2007), and are also potential regulators of muscle catabolism in cachexia (Keren *et al*, 2006). ERK appears upregulated in the muscle of animals bearing experimental tumours, and its inhibition corrects the loss of both muscle weight and function. Interference with ERK activation is also able to prevent the enhanced expression of the ubiquitin ligase/atrogin-1 involved in muscle catabolism (Penna *et al*, 2010). These observations suggest that treatments able to modulate or to interfere with MAPKs may be relevant to the prevention/correction of cancer cachexia.

Chapter 6 suggests that multiple pathways are likely to be involved in the pathogenesis of cancer cachexia and, in particular, appetite regulation, glucocorticoid signalling, and MAPK

activity regulation may have central roles in this process. Indeed, it is now well accepted that a complex interplay of mediators, such as hormones and cytokines, plays a role in the onset of cachexia, leading to muscle and adipose tissue depletion and loss of function (Durham *et al*, 2009). These findings are highly exciting in terms of isolating potential biomarkers of cachexia among components of the pathways described above. There is a possibility of developing biomarker sets based on the above pathways to predict predisposition to cachexia as well as indices of cachexia progression and/or response to treatment.

These findings also open up new possibilities in terms of identifying new targets for drug therapy. So far, individual treatments such as those aimed at improving anorexia, and blocking glucocorticoids have had mixed results in treating cachexia. If further studies prove that the pathways found in the study in Chapter 6 are indeed central to the development of cachexia, perhaps a multi-therapy approach against components of each pathway may prove more efficacious in treating cancer cachexia.

7.5 Future work and direction

One of the main difficulties in research involving cachexia is the lack of a specific phenotype. However, chapter 3 has demonstrated that CT scans can be used to derive an index of muscle mass to classify patients as sarcopenic or not using established cut-offs of skeletal muscle index of <7.26 kg/m^2 for men and <5.45 kg/m^2 for women. Sarcopenia and sarcopenic obesity have been linked with multiple relevant clinical outcomes in cancer patients. Further work would involve examining the potential relationship between patients' genotype and the propensity to sarcopenia and/or sarcopenic obesity in cancer.

Chapter 3 has re-iterated that weight-losing advanced cancer patients have an energy deficit of approximately 300kcal/day. This deficit and the likely associated reduction in protein intake are factors that may contribute to the development of sarcopenia. It has been shown that nutritional intervention alone cannot effectively treat cachexia. Many classes of drugs aimed at treating cancer cachexia have been evaluated with mixed results. Further randomised clinical trials are therefore required to investigate if early nutritional intervention coupled with additional therapy targeted at attenuating catabolism and/or improving anabolism, especially in pre-cachetic cancer patients, improves muscle mass and thus positively affects outcome.

This thesis chose to examine the cachexia phenotype based on weight loss using cut-offs set at >5%, >10% and >15% with and without the presence of systemic inflammation. Despite the limitation of the small sample size in the validation cohort of the candidate gene study in Chapter 6, the P-selectin (*SELP*) rs6136 polymorphism appears to be a promising biomarker in terms of determining susceptibility to cachexia. Future work would involve recruiting a larger cohort of patients to validate rs6136 and the other polymorphisms associated with cachexia found in Chapter 6.

Further experiments are also required to investigate the role and importance of P-selectin in the development of cancer cachexia. Recent work done by collaborators highlight evidence for the role of P-selectin in the development of cachexia by the induction of muscle atrophy in mice/rats either by intra-peritoneal injection of LPS or in rats bearing the MCA sarcoma. qPCR analysis of skeletal muscle RNA following either intra-peritoneal injection of LPS or in tumour bearing animals showed a striking upregulation of the P-selectin transcript. Furthermore, preliminary studies also indicate that P-selectin show a similar striking

upregulation (10-fold) two hours after intracerebroventricular (ICV) injection of interleukin-1 beta in mice (Braun *et al*, 2011). Acute and chronic infusion of IL-1β into the brain leads to muscle breakdown, anorexia, weight loss and negative nitrogen balance (Hill *et al*, 1996) and is a potential central mediator of LPS effects. Identification of P-selectin as a top early induced gene of the mouse/rat muscle atrophy program and the significant association of the rs6136 SNP in the P-selectin gene with wasting in cancer patients provide supportive evidence for the likely involvement of P-selectin in muscle wasting.

Does P-selectin thus play an important causal role in the development of cancer cachexia? Further work would involve the creation of knockout mice sans the P-selectin gene. If muscle mass is maintained in these animals following similar induction of the muscle atrophy program, then it will provide irrefutable evidence of the causal role of P-selectin in cachexia.

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Appendix A **Patient Information Sheet**

THE IDENTIFICATION OF GENETIC MARKERS OF CANCER-ASSOCIATED WASTING

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

The purpose of this research study is to find out if there is a genetic basis for people who have cancer to become wasted, lose weight and feel tired. This wasting is a significant problem for patients as it causes a reduction in both quality and quantity of life. In order to find out if there is any way to predict its development, we would like to see if wasting in cancer is related to the presence or absence of certain genetic markers.

Why have I been chosen?

You have been chosen because you have been diagnosed with oesophageal, gastric or pancreatic cancer. We aim to recruit 300 participants in total.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw at any time in the future and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that you receive.

What will happen to me if I take part?

If you take part, you will be asked to undergo a single blood test and have your weight and height measured. This will take place during a routine outpatient clinic appointment with your consultant. No extra appointments are required if you choose to take part in this study. We will also ask your permission for access to your clinical case notes and any CT scan images that are performed as part of your routine treatment.

- ***Blood test:*** The amount of blood taken is approximately the same as one teaspoonful (15ml), and it will be taken from your arm in the usual fashion.

What do I have to do?

Apart from attendance at the initial appointment, no other responsibilities are required from your participation.

What are the possible disadvantages and risks of taking part?

There are negligible disadvantages and risks of taking part. Minor discomfort may occur during blood sampling.

What are the possible benefits of taking part?

We cannot promise that the study will help you, but the information we receive might help improve the treatment of patients with cancer and cancer-associated weight loss.

What happens when the research study stops?

Following your initial appointment, no further appointments are required. However, if any of your blood, or DNA samples remain, we would ask your permission to store these samples (in anonymised form) in the University of Edinburgh so that we can consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the studies appropriate).

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this, including contact details, is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Part 2

What if relevant new information becomes available?

If any new treatment for cancer or cancer-associated wasting becomes available during the time of the study, it will not be withheld from you because of your participation in this study. Furthermore, if you require any other treatment for cancer during the course of the study (e.g. chemotherapy or radiotherapy), it will not be withheld from you because of your participation in this study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. All information that was collected during the time of your participation in the study will be destroyed. Any stored blood or DNA samples that are identified as yours will also be destroyed.

What if there is a problem?

- **Complaints:** If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.
- **Harm:** In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence, then you may have grounds for a legal action for compensation against the University of Edinburgh but you may have to pay for your legal costs. The normal National Health Service complaints mechanism will still be available to you.

Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. However, we would like to inform your GP of your involvement in this study but we will require your permission to do this. All other information about you which leaves the Royal Infirmary of Edinburgh will have your name and address removed so that you cannot be recognised from it.

Blood and DNA samples collected during the study may be transferred for the purpose of analysis to associated researchers within the European Economic Area. However, all samples will be anonymised prior to sending and therefore you will not be identifiable.

What will happen to any samples I give?

A portion of the blood sample will be immediately analysed by the Department of Biochemistry at the Royal Infirmary of Edinburgh or Western General Hospital. The remainder of the blood sample will be transferred to the University of Edinburgh for analysis. The only individuals who will have direct access to these samples will be the members of the study research team. DNA will be extracted

from the blood sample at the University of Edinburgh. A small portion of the DNA will be sent from the University of Edinburgh to our collaborators at the Norwegian University Science and Technology, Trondheim, Norway, for genetic analysis. The samples that are sent to Norway will already be anonymised so that you will not be identifiable from them.

Following all of these different analyses, if any of the samples remain, we would ask your permission to store these samples (in anonymised form) in the University of Edinburgh so that we can consider them for future research studies (if a local Ethics Committee deems the studies appropriate). All of the DNA sent to Norway will be 'used up', and therefore, there will be none left over which will require storage overseas. Professor Kenneth Fearon, Professor of Surgical Oncology, will act as custodian for any stored samples. The only other individuals who will have direct access to the stored samples will be the members of the research team behind the current study.

Will any genetic tests be done on the samples that I give?

Yes. There will not be any implications, healthcare or otherwise, from the genetic study. The genetic markers that we aim to test for have not been shown to be linked with or have any association with inheritable risk for any known diseases. Therefore, we will not normally feedback any results to you.

What will happen to the results of the current research study?

The results of this study will be published in medical journals, reports and textbooks. You will not be identifiable in any report/publication or report unless you have specifically consented to release such information.

Who is organising and funding the research?

The research is being organised and sponsored by the University of Edinburgh. The research is being funded by the European Commission.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by Lothian Research Ethics Committee. This study has also been reviewed by members of the scientific committee of the European Commission.

Contact details

You may contact me (the main researcher) directly by telephoning 0131 242 6520 for further information at any time. Alternatively, you may contact Prof. Stephen Wigmore, Consultant Surgeon in the Department of Surgery, who is acting as an independent advisor – contact 0131 242 3615.

Many thanks for your time.

Mr. Benjamin Tan
Clinical Research Fellow
Department of Surgery
Royal Infirmary of Edinburgh

Appendix B **Patient Consent Form**

CONSENT FORM

THE IDENTIFICATION OF GENETIC MARKERS OF CANCER-ASSOCIATED WASTING

1. I agree to take part in the above-titled study. ☐

2. I agree for my blood to be taken for the purpose of DNA analysis. ☐

3. I confirm that I have read and understand the information sheet dated June 2008 (version 2.1) for the above study. I have had the opportunity to consider the information and ask questions, and I have had these answered satisfactorily. ☐

4. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

5. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from Lothian NHS Trust University Hospitals Division, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

6. I agree to my GP being informed of my participation in the study. ☐

7. I agree to the storage of samples taken during the course of this study so that they may be considered for use in future research studies (pending a favourable ethical opinion by Lothian Local Research Ethics Committee). ☐

Name of Patient	Date	Signature
Researcher	Date	Signature

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REVIEW ARTICLE

Biomarkers for cancer cachexia: is there also a genetic component to cachexia?

B. H. L. Tan · D. A. C. Deans · R. J. E. Skipworth · J. A. Ross · K. C. H. Fearon

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Abstract
Introduction Cancer cachexia is a severe debilitating disorder, which causes significant morbidity and mortality. In clinical practice, cachexia is often not treated until a late stage, when therapeutic options are limited.
Objective It is therefore of great interest to analyse early biomarkers of this syndrome.
Conclusion In this review article, we summarise recent biomarkers found in various body compartments. We also explore the likelihood of a genetic predisposition to cachexia and focus on the potential role of single nucleotide polymorphisms in genes coding for pro- and anti-inflammatory cytokines, and 'atrogenes' associated with wasting in skeletal muscle.

Keywords Cachexia · Cancer · Biomarkers · Genetics · Polymorphisms

Introduction

Cachexia is a wasting condition that manifests itself in several life-threatening diseases, including cancer, AIDS, congestive heart failure and sepsis [3, 41]. Patients exhibit a loss of both adipose tissue and lean body mass [17], which is resistant to conventional nutritional support [45]. Cachexia is typically characterised by severe weight loss, anorexia, early satiety, weakness, anaemia and oedema [17]. The cachectic state is particularly problematic in cancer, typified by poor prognosis and often associated with a lower response to chemotherapy and radiotherapy than might be expected [43]. Patients are also more likely to report decreased quality of life (QoL) scores [18]. More than half of cancer patients suffer from cachexia, and it is responsible for death in up to 20% of cases [43]. Cachexia is therefore a significant cause of morbidity and mortality in cancer patients.

Once the patient becomes immobilised in advanced cachexia, there is little room for purposeful therapeutic intervention. Furthermore, it is questionable whether medical intervention is justified at this very late stage in the patient's life. In terms of cachexia therapeutics, it is therefore important to consider the overall journey the patient makes through the cachexia process and perhaps plan intervention at an early stage.

Although the mechanism for cancer cachexia remains largely unresolved, it has long been considered to be the result of a variety of interactions between the host and the tumour [39]. The presence of a tumour results in the initiation of a host inflammatory response mediated by tumour-derived pro-inflammatory cytokines [14]. Systemic inflammation then initiates a reprioritisation of protein metabolism with induction of the acute phase response and mobilisation of fat reserves. This, together with pro-cachetic factors, secreted by the tumour, promotes protein and fat breakdown [21, 46]. Other overlapping pathways such as upregulation of protein degradation pathways [e.g., the ubiquitin-proteasome system (UPS)] and dysregulation of the dystrophin glycoprotein complex (DGC) facilitate muscle catabolism [1, 42]. Finally, activation of neuroendocrine pathways may also contribute to hypermetabolism and increased catabolism [2]. Based on these mechanisms, predictive or early biomarkers of cachexia could be developed, which would aid in the selection of patients for early therapeutic intervention.

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Biomarkers of cachexia

There is substantial literature relating to a variety of potential biomarkers for cachexia derived from different body compartments, including plasma, urine, tumour, skeletal muscle and the patient's genome (Fig. 1). This review aims, primarily, to consider the great potential of isolating markers for cachexia in the patient's genome. To make this review more complete, we will also briefly summarise some of the aforementioned phenotypic biomarkers found in other body components.

Plasma

Plasma is easily sampled and, therefore, an important compartment to study for potential biomarkers of cachexia. The positive hepatic acute phase reactant, C-reactive protein (CRP) has been shown to be a robust marker of systemic inflammation in cancer patients and has been associated with the presence of anorexia, hypermetabolism, accelerated weight loss and shortened survival [16, 26, 51]. CRP has been studied in a wide variety of tumour types and has been incorporated into a three-factor mathematical definition of cachexia for patients with pancreatic cancer [18]. CRP is therefore a key potential biomarker for cachexia.

The plasma concentration of a variety of pro-inflammatory cytokines has been evaluated in numerous studies in human subjects in relation to the development of cancer cachexia, including tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), IL-6 and interferon gamma (IFN-γ) [2, 8, 29, 48]. These studies, unfortunately, have not given a clear, reproducible pattern whereby the plasma concentration of any individual cytokine can be related to the development of cachexia. Controversy has surrounded the use of different cytokine assays, their variable sensitivity, the short half life of cytokines and the presence of natural cytokine inhibitors.

Recently parathyroid hormone-related peptide (PTHrP) has been described as a potential mediator of cancer cachexia. PTHrP has been shown to be present in approximately 17% of patients with gastro-oesophageal malignancy (in the absence of hypocalcaemia) and to be associated with markers of systemic inflammation and adverse prognosis [13]. Thus, PTHrP might well serve as a useful biomarker, but this awaits confirmation in other types of malignancy.

In terms of the neuroendocrine axis, insulin resistance and hypercortisolaemia have been documented in the cachectic cancer patient [30, 35]; however, these have not been studied in detail in terms of the early cachectic process. Hypogonadism in males is associated with sarcopenia and has been

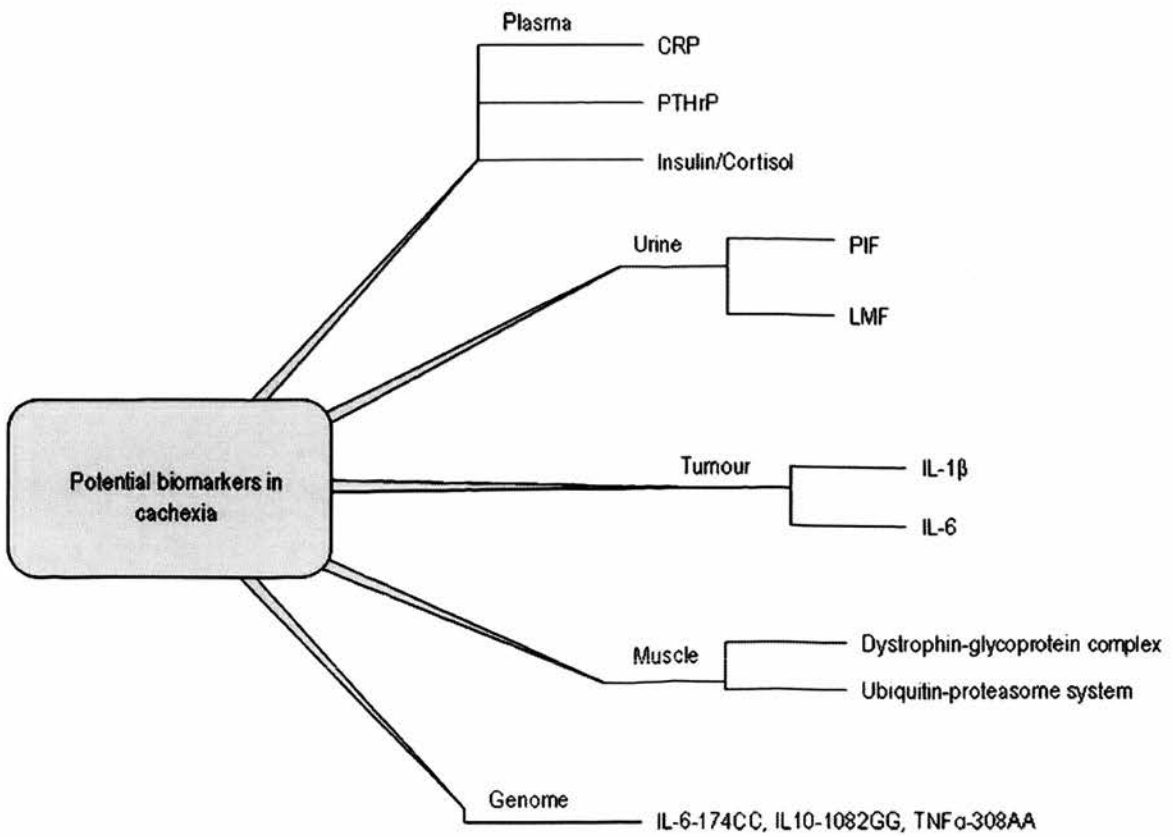

Fig. 1 Potential biomarkers for the development of cancer cachexia

shown to be highly prevalent in male patients with advanced cancer [11]. However, it is not clear just how independent of systemic inflammation the development and effects of hypogonadism in advanced cancer might be. Moreover, it is not clear how sex hormone levels influence muscle mass maintenance in female patients with cachexia. Angiotensin II has been shown to produce muscle catabolism and weight loss in murine models, and these effects are attenuated by angiotensin-converting enzyme (ACE) inhibitors [33]. Angiotensin II may therefore be an interesting marker for cachexia.

Finally, other potentially useful plasma markers include peptides that are involved in the control of food intake such as leptin and ghrelin [37, 53]. Whilst both factors have been implicated in the anorexia component of the cachexia syndrome, the complexity of their interaction with other factors (e.g., end-organ sensitivity) that control appetite makes them unlikely as robust biomarkers that can be used in isolation.

Urine

Compared with plasma, urine represents a relatively simple matrix to look for novel biomarkers. Indeed, it was from urine that a potential human homolog of proteolysis-inducing factor (PIF) was isolated initially. PIF is a 24-kDa glycoprotein produced by tumour cells in both mice and humans that has been hypothesised to be responsible for cancer cachexia [46]. A putative human homolog of murine PIF has been found in many human tumour types, including breast, ovarian, pancreatic and colorectal cancers [44]. PIF appeared to be present in patients with tumour-related cachexia but was absent from normal subjects, patients with weight loss due to trauma and cancer patients with little or no weight loss [5]. Mice administered with PIF showed rapid muscle catabolism, whilst PIF-induced weight loss in mice is reversed with anti-PIF monoclonal antibodies [5]. Recently however, the specificity of the antibody used to isolate the human homolog of PIF has been called into question, and this has hampered isolation of bioactive PIF for confirmation of its role in human biology [50].

Lipid-mobilising factor (LMF) is a 43-kDa lipolytic factor derived from both tumour and brown adipose tissue. It has also been isolated from urine of both cachetic cancer patients and cachetic mice [9, 21]. LMF is thought to be responsible for atrophy of adipose tissue in cachetic patients [9]. One study had found that cancer patients with weight loss had detectable concentrations of LMF in their urine, whilst cancer patients without weight loss did not [47].

Tumour

The systemic inflammatory response frequently observed in patients with cancer cachexia is thought to originate from within the tumour [14, 27]. As such, inflammation can be generated by a tumour mass either from constitutive pro-inflammatory cytokine production by the tumour cells or via activation of host microphages or more likely by a combination of the two. From studies in human pancreatic cancer, it has been suggested that upregulation of IL-6 may be a key initiating factor of cachexia from within the tumour [27]. However, a recent study into oesophageal cancer has suggested that IL-1β is perhaps of more importance [14]. Thus, it is not clear that there is a single tumour-associated cytokine, which could serve as a generic biomarker. However, it may be that within a single tumour type, the pattern of cytokine expression might provide a useful signature. Clearly, however, not all patients have tumour biopsies available for such characterisation, and thus there, are clear practical limitations to this approach.

Muscle

Various aspects of the molecular mechanisms of muscle atrophy or hypertrophy have now been described in detail [32]. It would appear that in cancer cachexia, there is suppression of the hypertrophy pathways and upregulation of the atrophy pathways [38]. The hypertrophy pathways seem to predominate and in particular activation of the UPS, which is the major catabolic pathway in cancer cachexia [25]. Overactivation of UPS has been documented in rodent models of cancer-associated muscle wasting [23]. Research on human subjects with cancer has also demonstrated UPS overactivation [4]. Activation of the ubiquitin proteosome pathway has been ascribed in part to the presence of systemic inflammation [15]. Recent data has suggested that such systemic inflammation may act in part through dysregulation of the DGC [1]. The DGC is a muscle-specific protein manifold that anchors muscle fibre membranes in place and prevents them from being torn by shear forces produced during muscle contraction. The dysregulation of DGC has been shown to correlate positively with weight loss in patients with gastro-oesophageal adenocarcinoma [1]. It remains, however, difficult to use skeletal muscle as an early biomarker, as at present, this would require percutaneous biopsy or open biopsy.

Rationale for a genetic component to cachexia

Based on current knowledge of demographic and clinical factors, it is not possible to predict, for any given cohort of patients, who will develop cancer cachexia and who will not. Moreover, even with all the potential biomarkers described above, it is not possible to predict accurately who will develop cachexia quickly vs those who may develop the syndrome at a

slower pace. Such variation may, in part, be due to the patient's genotype rather than the tumour phenotype. The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome (e.g. systemic inflammation). However, it must be said that, unlike other common diseases where twin studies have suggested a clear heritable component to the disease (e.g. Crohn's disease, etc), no such studies support a heritable component to cachexia.

Genotyping cachexia

In terms of linking a patient's genotype with a pro-cachectic phenotype, there are two basic methods. One could undertake human linkage studies with genome-wide linkage scans to identify quantitative trait loci. This methodology is complex, expensive and, in some instances, is limited by the current development of bioinformatics. On the other hand, one could undertake analysis of specific candidate genes. In this regard, the commonest form of analysis is allelic association studies using single nucleotide polymorphisms. Single nucleotide polymorphisms (SNPs) are specific positions within the genome that are represented by more than one nucleotide in a population. The definition of polymorphism is that it has an allele frequency >1%. SNPs occur in approximately one in every thousand bases. Only 4% of SNPs are within genes [40].

An allelic association is where there is non-random distribution of allele frequencies with respect to disease status. There are a variety of problems with allelic association studies; in particular, many studies have suffered from small sample size. There have also been issues concerning phenotype misclassification (which is particularly relevant to cancer cachexia where there is no agreed definition of the syndrome), epistatic interaction and undetected genomic heterogeneity in the underlying population. Taken together, these limitations are exemplified by the fact that in a recent review of 600 published reports of allelic association with common disease phenotypes, only 6 of 166 associations subjected to multiple evaluations were confirmed consistently [22].

Selection of candidate genes

Systemic inflammation

Pro-inflammatory cytokine genotype

In studies linking cytokine genotype to the outcome of acute sepsis, a variety of important principles have emerged. One key question is whether the product of the gene plays an important role in the pathogenesis of the disease. Certainly in relation to systemic inflammation and its fundamental role in cachexia, this can be answered affirmatively for pro-inflammatory cytokines [8, 29]. The second important issue is whether the gene polymorphism produces a relevant alteration in the level or function of the gene product. Fortunately, there are firmly established functional sequelae for a variety of polymorphisms in connection with pro-inflammatory cytokines [19, 31, 52]. It would therefore seem reasonable to consider such polymorphisms as candidate biomarkers in human cancer cachexia.

A small number of preliminary studies have related single nucleotide polymorphisms in a variety of pro-inflammatory cytokine genes to the presence of systemic inflammation, cachexia and accelerated demise in advanced cancer [6, 12]. These studies will now be reviewed in detail.

Deans et al. [12] have recently evaluated the role of a variety of pro-inflammatory and anti-inflammatory cytokines in the genesis of systemic inflammation and adverse prognosis in patients (n=203) with oesophago-gastric cancer. The IL-6 174CC polymorphism was found to be associated with both systemic inflammation and high levels of intra-tumoural cytokines. In turn, the presence of systemic inflammation was related to adverse prognosis. The 1082GG polymorphism in the IL-10 gene was also related to systemic inflammation and adverse prognosis. Thus, polymorphisms in the IL-6 and the IL-10 genes were candidates for disposition to cachexia. In contrast, the 308AA polymorphism in the TNFα gene was associated with adverse prognosis but did not relate to systemic inflammation or intra-tumoural cytokine levels. Finally, the 511CC polymorphism on the IL-1β gene was found not to be associated with tumour cytokine levels, systemic inflammation or adverse prognosis. The main drawback of this type of study is that, although a potential mechanism (systemic inflammation) and effect (shortened survival) of cachexia was examined, the actual development of wasting was not documented. In contrast, a recent study of advanced gastric cancer patients (n=214) from China [54] demonstrated that patients with the IL-1β+3954 T allele showed an increased prevalence of cachexia (relative risk of developing cachexia increased by a factor of 2.5).

Patients with pancreatic cancer have a very high incidence of cachexia and have been studied in relation to polymorphisms within both the IL-1β gene and TNF gene [6, 7]. TNF-308 and TNFB and were not found to relate to enhanced peripheral blood mononuclear cell IL-1 production, systemic inflammation or adverse prognosis. In contrast, the IL-1β 2/2 polymorphism was related to all three. There was, however, no specific measurement of cachexia in these studies.

Finally, in relation to COPD cachexia, a recent study has demonstrated that polymorphisms of the IL-1β-511 SNP are associated with the development of cachexia [10]. However,

polymorphisms on the TNFα gene, the IL-10 gene and the IL-6 gene did not relate to cachexia or the development of systemic inflammation.

Anti-inflammatory cytokine genotype

If there are polymorphisms that lead to a pro-inflammatory cytokine genotype, it would be logical to expect that certain polymorphisms would favour an anti-inflammatory cytokine genotype.

There are studies that demonstrate increase of anti-inflammatory cytokines in the plasma due to polymorphisms in the anti-inflammatory cytokine genes [20, 34]. However, there is a paucity of studies linking these polymorphisms to cachexia or resistance to cachexia.

Available studies have looked at survival and not the actual development of cachexia. Vuoristo [49] examined 108 patients with melanoma and found that the IL-10 polymorphisms -1082A, -819T and -592A formed a haplotype, which confers longer survival. In patients with renal cell carcinoma, a study (n=80) found that individuals homozygous for the IL-4 polymorphisms -589C and -33C formed a haplotype that increased survival duration [24].

Muscle atrophy genes ('atrogenes')

Recent studies of the Belgian Blue strain of cows, which develop grossly enlarged muscles, have revealed this phenotype to be due to a mutation in the myostatin gene [28]. A similar syndrome in children has also been linked to a similar mutation [36]. This has highlighted the potential for genetic variation in either the hypertrophy or atrophy pathways within skeletal muscle to account for a predisposition either to the presence of a high or low muscle mass. Such genes are therefore candidates for a pro-cachectic genotype. However, although a number of array studies have linked differential expression of these genes with models of muscle atrophy [32], there are no specific studies in relation to human cancer cachexia.

Conclusion

There are a wide range of potential biomarkers (derived from different body compartments) for the cachexia syndrome. Probably the most robust at present is the inflammatory marker CRP measured in plasma or serum. As such, there is a reasonable argument to explore genetic predisposition to systemic inflammation and the development of cancer cachexia. Other target genes may be involved such as those of skeletal muscle atrophy pathways. Different patterns of genes are likely to be involved in different tumour types associated with cancer cachexia and in different benign diseases associated with the cachectic process.

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Cachexia: prevalence and impact in medicine

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Purpose of review

Cachexia is a progressive deterioration of body habitus associated with certain chronic diseases (e.g., cancer, chronic obstructive pulmonary disease, chronic heart failure, and chronic kidney disease). The aim of this article is to describe the prevalence and impact of cachexia (and precachexia) in such patients.

Recent findings

Owing to the wide spectrum of clinical presentation and lack of an 'all-inclusive' definition, it is difficult to estimate the true prevalence of cachexia. Perhaps 2% of the population suffer from precachexia (characterized by weight loss in association with a chronic disease). The significant increase in obesity of the general population (which can mask significant muscle wasting) confounds such simple estimates of the true prevalence of cachexia. In contrast, a multidimensional characterization of the cachectic state (including weight loss, reduced food intake, and systemic inflammation) may be more meaningful in terms of altered clinical outcomes. Such a multidimensional view of cachexia has been shown to impact on patients' survival and quality of life and therefore constitutes a major public health issue.

Summary

There is a high prevalence of (pre)cachexia in patients with chronic diseases. The cachexia syndrome is probably less frequent but has a significant impact in terms of morbidity and mortality.

Keywords

cachexia, chronic diseases, impact, prevalence, reverse epidemiology

Curr Opin Clin Nutr Metab Care 11:400–407

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. . .the shoulders, clavicles, chest and thighs melt away. This illness is fatal. . .

(Hippocrates 460–370 BC)

Introduction

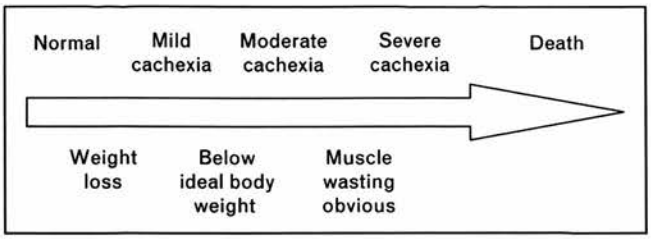
Cachexia is a complex syndrome that combines weight loss, loss of muscle and adipose tissue, anorexia, and weakness [1]. The origin of the term 'cachexia' is from the Greek words kakós (bad) and hexis (condition or appearance) and throughout medical history has been associated with the gravely ill patient.

Cachexia significantly impairs quality of life (QoL) and response to treatment and is associated with increasing morbidity and mortality. Cachexia typically manifests in chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), and chronic kidney disease (CKD) [1]. The aim of this article is to describe the prevalence of cachexia (and precachexia) in such chronic diseases and the impact of cachexia on patients' function and survival.

Precachexia versus the cachexia syndrome

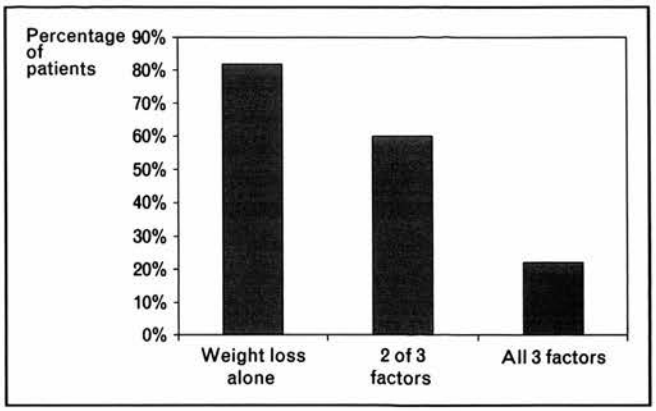
Every journey begins with a single step and it is important to remember that cachexia represents a spectrum. Patients may first notice simple weight loss and then progress through degrees of severity to the point where they are depleted of energy reserves (fat), have gross muscle wasting, are immunocompromised, and will die primarily as a result of these issues (Fig. 1). When trying to describe cachexia and its impact, the initial phase (precachexia) may have little impact, whereas the advanced phase (cachexia syndrome) will impact on both quality and quantity of life. In a recent study of patients with advanced pancreatic cancer [2], we have sought to define the features of cachexia that impact on patients' function and survival. Three key features were identified: weight loss (>10%), systemic inflammation (C-reactive protein >10 mg/l), and reduced food intake (<1500 kcal per day). When patients were grouped according to weight loss alone, nearly 80% fell into this category yet this did not define patients with altered body composition or reduced subjective functional health status. In contrast, when grouped according to the presence of all three factors, patients demonstrated reduced lean body mass, reduced objective and subjective indices of function,

Figure 1 The cachexia journey



Not all patients progress along the entire pathway.

Figure 2 Prevalence of cachexia in patients with unresectable pancreatic cancer according to components of a multidimensional definition of cachexia



The three-factor profile of cachexia consists of weight loss (>10%), reduced food intake (<1500 kcal per day), and presence of systemic inflammation (C-reactive protein >10 mg/l). Adapted from [2].

and reduced health status. Importantly, the number of patients who fulfilled the three-factor profile was only about 20% of the total population (Fig. 2). Thus, although the prevalence of cachexia may be less when considered in a more multidimensional form, its impact on the patient is more devastating.

Equally not all patients will progress down the full cachexia spectrum. Some may die of their primary disease before they develop advanced cachexia, others may stabilize as a result of treatment of their primary disease or due to other clinical factors that preclude further progression. Such heterogeneity makes it particularly difficult to target prophylactic therapy successfully. Prophylaxis would be best initiated in the precachexia phase, yet there are few robust predictors to guide such strategy. The situation is made even more complex when one considers that a substantial portion of the patients 'at risk' will be obese initially (see later). Weight loss in obese patients (or any group) may simply reflect increasing severity of the underlying disease. As such, early weight loss will indicate increased risk of morbidity/mortality, but it will not necessarily indicate those who are going to develop advanced cachexia.

Prevalence of precachexia

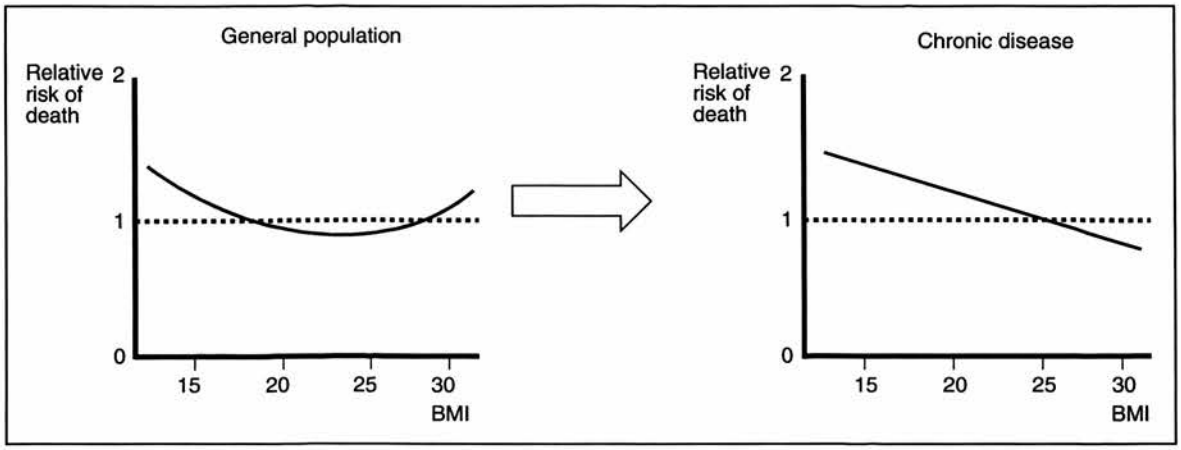
Considering the United States (population of approximately 290 million) as a representative country for the Western World, the approximate prevalence of the four major chronic diseases associated with cachexia are presented in Table 1. Together some 30.5–34.5 million patients suffer from cancer, COPD, CHF, and end-stage renal failure (ESRF). Using recently published definitions of (pre)cachexia [3–6], approximately 5–5.7 million patients are likely to suffer from (pre)cachexia, giving a prevalence in the general population of approximately 2%. Clearly this estimate is open to many confounders that make it either an overestimate (e.g., patients having both COPD and cancer) or an underestimate (failure to include children or rarer diseases associated with cachexia). More fundamental issues affecting the accuracy of this estimate include the lack of a uniform definition for cachexia and considerations whether the general increase in obesity in the general population is leading to inappropriate patients being considered as having cachexia.

Table 1 Prevalence of (pre)cachexia in United States (total population ≈290 million)

	Number in United States (millions)	Risk index	Number at risk (millions)
Cancer	11–13	WL > 5%[a]	2.4–2.9
COPD	15–16	FFMI: men ≤16, women ≤15[b]	2–2.1
CHF	4–5	WL > 6%[c]	0.5–0.6
ESRF	0–4	MIS[d]	0.1
Total	30.5–34.5		5–5.7

CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; ESRF, end-stage renal failure; FFMI, fat free mass index; MIS, malnutrition inflammation score; WL, weight loss.
[a] Data from [3].
[b] Data from [4].
[c] Data from [5].
[d] Data from [6].

Figure 3 Risk factor paradox in chronic disease



When patients develop certain chronic diseases (e.g., cancer, COPD, CHF or ESRF), the risk of mortality can be paradoxically lower in high BMI categories. CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; ESRF, end-stage renal failure; LBM, lean body mass. Adapted from [7••].

Obesity, reverse epidemiology, and the nature of the risks associated with weight loss

In epidemiology of the general population, the relationship between the risk of mortality and BMI is U-shaped with increased risk associated with either very low BMI (cachexia) or very high BMI (morbid obesity). However, emerging data indicate that once patients have developed certain chronic diseases (e.g., cancer, COPD, CHF, ESRF), conventional risk factors such as obesity are associated paradoxically with better survival [7••] (Fig. 3).

There is no doubt that patients with the chronic diseases normally associated with cachexia are becoming increasingly obese. For example, despite a predicted prevalence of weight loss affecting nearly 50% of patients [8], those newly diagnosed with incurable cancer commonly have a mean BMI higher than 25 [9]. When such obese persons lose weight, their reduced risk of mortality may move back towards the mean, but the nature of their risk is surely different from that of the severely wasted individual (Fig. 4) and the specifics of therapeutic intervention are also likely to be different. Indeed, in the obese population, it might be said that if the increased risk of early weight loss reflects increased severity of the patients' primary disease, then nutritional intervention would have little role in the management of such patients.

However, the above discussion represents an oversimplification. It is well known that in obesity, a small degree of weight loss can mask a proportionately higher loss of skeletal muscle mass [10]. This has been shown recently by Baracos and coworkers (personal communication) who demonstrated that in advanced cancer patients with a mean BMI of 25.5, the prevalence of sarcopenia in those below the mean BMI approaches 80%. Separating out 'who' is at risk from 'what' remains a major challenge for the characterization of patients at risk from cachexia.

Prevalence of (pre)cachexia in specific diseases

An estimate of the extent of (pre)cachexia in various chronic diseases is presented below.

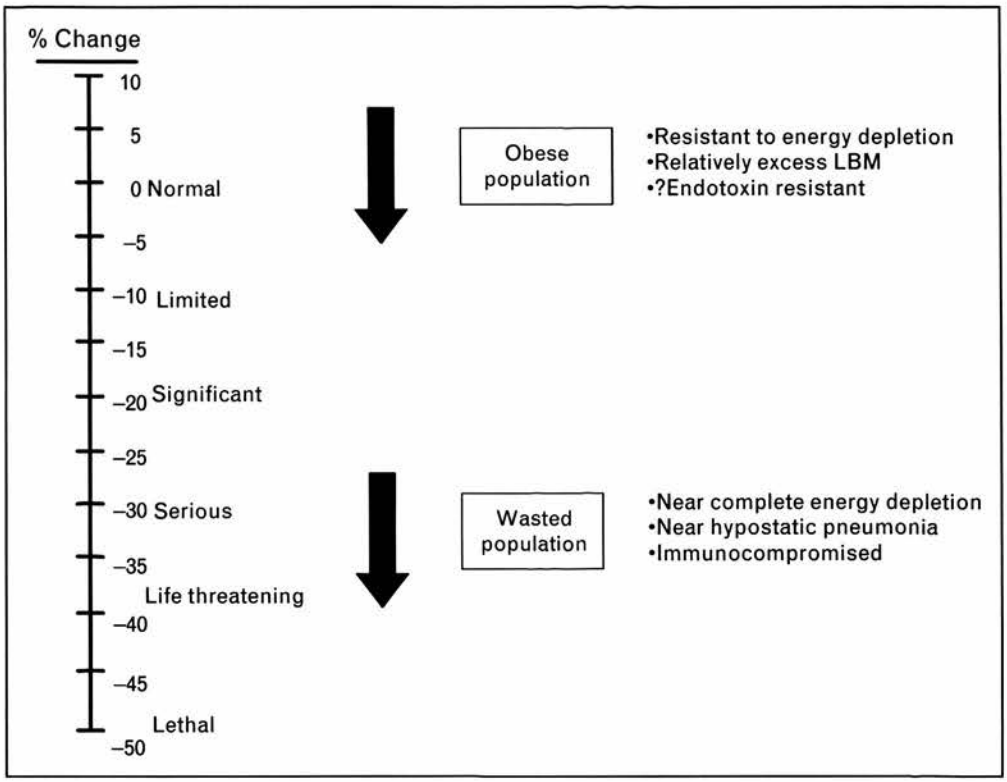
Cancer

Approximately one quarter of all deaths in Western Society are due to cancer. Half of all patients with cancer lose some body weight; one third lose more than 5% of their original body weight and up to 20% of all cancer deaths are caused directly by cachexia (through immobility, cardiac/respiratory failure) [11•]. The incidence of weight loss upon diagnosis varies greatly according to the tumour site (Fig. 5). The greatest incidence of weight loss is seen among patients with solid tumours, for example, gastric, pancreatic, lung, colorectal, and head and neck [12]. Eighty percent of the patients with pancreatic cancer have at least 10% weight loss at diagnosis and the cachexia syndrome is present in 25% [2]. The overall prevalence of weight loss in cancer patients may rise as high as 86% in the last 1–2 weeks of life [8].

Chronic heart failure

Approximately 1.5–2% of the population in the United States suffer from CHF [13]. The prevalence of CHF rises nearly exponentially with age, from 0.02 per 1000 population per year in those aged 25–34 years to 11.6 in those aged 85 years or older [14•]. Although every

Figure 4 Physiological risk from losing weight is related to initial body mass



Obese patients who lose 10% of their body weight may not have significant nutritional sequelae, but their mortality risk may increase as a result of their primary underlying disease. In contrast, wasted patients who lose 10% of their body weight may die as a result of protein-calorie malnutrition.

clinician is familiar with the classical 'skin-and-bone' appearance of a small group of patients with end-stage mitral valve disease, modern drug trials have revealed a potentially greater population of cardiac patients at risk from weight loss.

Using a cut-off of more than 7.5% weight loss over a period of at least 6 months, 28 out of 171 patients (16%) with CHF have been diagnosed as 'having cachexia' in a pilot study [15]. The observed weight loss in these 28 patients ranged from 9 to 36%. In a retrospective analysis of the SOLVD treatment trial [5], 702 of 1929 CHF patients (36%) with a weight loss of more than 6% were identified as being at increased risk of mortality. Unfortunately, these studies probably only reflect the prevalence of precachexia (based on weight loss). So far, there is no longitudinal nutritional data to record the proportion of CHF patients that complete the 'cachexia journey'.

Chronic obstructive pulmonary disease

There are approximately 15 million patients with COPD in the United States [16]. Weight loss in patients with COPD is associated with muscle weakness, diaphragmatic dysfunction, respiratory failure, poor QoL and death [17].

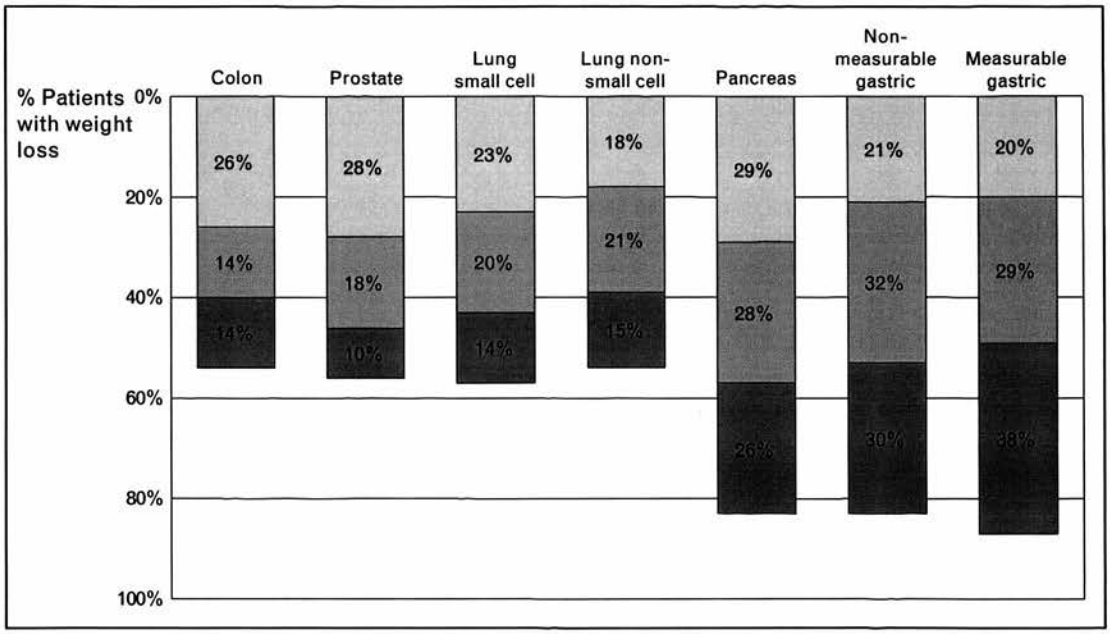
In a study of 389 patients with COPD [18], 27% were found to have nutritional depletion [i.e., BMI $\leq$21 kg/m^2 and/or fat free mass index (FFMI) $\leq$15 kg/m^2 (women) or $\leq$16 kg/m^2 (men)]. Using the same cut-offs to define cachexia, Schols *et al.* [4] showed that the prevalence of cachexia is much higher in severe COPD [Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) stage IV]. In a more recent study [19], 33% of patients with COPD were found to have cachexia (weight loss >7.5%). However, the interpretation of these prevalence values is limited by the use of simple weight loss in a population that is often obese.

COPD is a unique disease associated with cachexia, as there is grade A evidence to show that the cachexia syndrome can be treated successfully as part of a multimodal rehabilitation programme [20].

Chronic kidney disease

It is estimated that over 1.1 million patients with ESRD currently require maintenance dialysis worldwide [21].

Figure 5 Frequency and severity of weight loss in patients with advanced cancer attending for outpatient chemotherapy



The incidence of weight loss is particularly prominent in solid tumours of the upper gastrointestinal tract and lung. □ 0–5%; ▨ 5–10%; ■ >10%. Derived from [12].

This number is increasing at a rate of 7% per year, and is expected to exceed 2 million by 2010 [21].

In patients with advanced CKD and those on dialysis, there is a high prevalence of protein-energy malnutrition of up to 40% or more [22]. The term malnutrition–inflammation–cachexia syndrome (MICS) has been coined to reflect the high prevalence of malnutrition, inflammation, and wasting in patients with CKD [23]. MICS is reported to have a prevalence of 30–60% [24••]. It is, however, unclear to what extent the increased risk of mortality for patients with MICS comes from systemic inflammation *per se* versus any specific nutritional deficit.

Impact of cachexia

The following section describes the impact of cachexia in terms of increased morbidity and mortality, as well its socioeconomic consequences.

Impact on survival

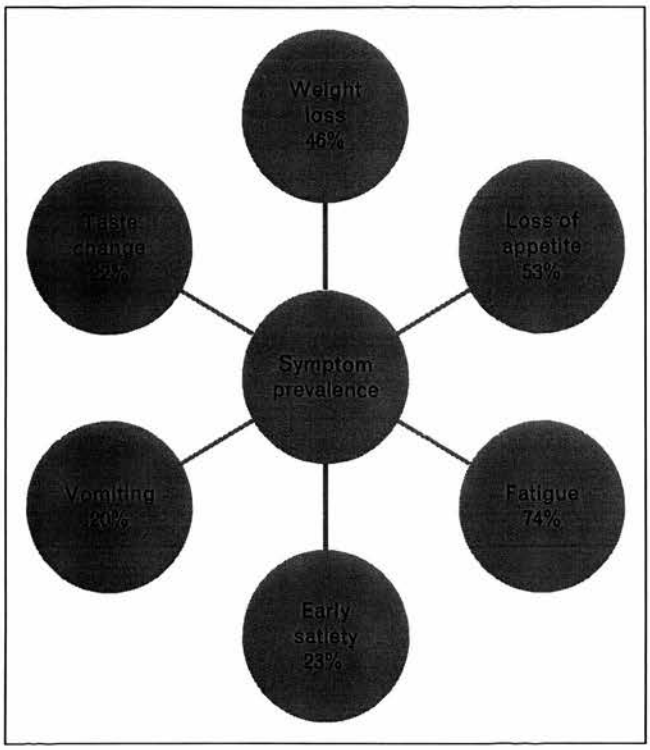
Based on the three-factor profile (weight loss, reduced food intake, and systemic inflammation) of cancer cachexia syndrome, we have noted that pancreatic cancer patients who met at least two of the components had a significantly worse prognosis [2]. Alternatively, in a study of patients with oesophageal cancer, we have found that weight loss of more than 2.75% per month is an independent prognostic indicator of decreased survival [25]. Paradoxically, high levels of absolute weight prior to diagnosis and substantial weight gain following diagnosis/treatment are associated with decreased survival in patients with breast cancer [26]. Thus, the impact of cachexia on cancer survival and the factors that contribute to such risk are probably tumour-type specific.

In a group of 171 patients with CHF, Anker *et al.* [15] demonstrated that the 'cachectic state' is an independent risk factor for poor outcome with a mortality rate after 18 months of 50% compared with 17% in patients without cachexia. In patients enrolled in the SOLVD trial ($n = 1929$), the poor outlook for weight-losing CHF patients was confirmed. Weight loss of more than 6% at any time was the strongest predictor of impaired survival [5].

In patients with COPD, survival is significantly decreased in patients with cachexia and muscle atrophy [4]. A low FFMI appears to be an independent prognostic factor leading to reduced survival [4].

Kalantar-Zadeh *et al.* [6] have developed a quantitative score as an indicator of MICS. The malnutrition inflammation score (MIS) utilizes seven components of the conventional Subjective Global Assessment of Nutrition (SGA) and combines it with three new elements [BMI, serum albumin, and total iron binding capacity (TIBC)]. In 378 patients, those who were in the highest quartile for MIS, survival was significantly reduced [6]. A more recent study confirmed that low BMI is associated with greater

Figure 6 Symptom prevalence in patients with incurable cancer ($n = 25\,074$)



Derived from [8].

mortality in patients with CKD not yet on dialysis therapy [27].

Impact on quality of life

Cachexia has a detrimental effect on a patient's QoL. Patients with cancer cachexia report altered body image, which impacts their emotions, spirituality, relationships, and social functioning. Lives are restricted and isolated, which is compounded by emotional distancing by carers and healthcare professionals [28•]. These patients also experience anorexia and increased fatigue (Fig. 6) [2,8]. Overall, this results in decreased performance status and QoL indices [29•]. COPD, CHF, and CKD patients with cachexia report similar decrease in QoL [30••,31,32].

The devastating effect of cachexia is further characterized by considering the free-living physical activity of patients. We have recently characterized patients using an electrical activity monitor worn over a period of 1 week. Patients with cancer cachexia demonstrate a 40% reduction in the level of physical activity [33].

Impact on treatment

Cachexia has a significant impact on cancer treatment. DeWys *et al.* [12] defined weight loss of more than 5% prior to the onset of chemotherapy as the defining point for risk of poor response to therapy and shortened survival. A separate study in patients with lung cancer showed that patients with weight loss more frequently failed to complete at least three cycles of chemotherapy and had decreased survival duration [34].

Moderate exercise training has been shown to improve exercise capacity in patients with CHF by reversing muscular metabolic abnormalities and atrophy as well as impaired blood flow and neurohormonal abnormalities [35]. However, due to inability to mobilize, the benefits of exercise are probably not an option for patients with (at least) severe cachexia.

Maintenance treatment for many patients with COPD consists of corticosteroids. In patients with established pulmonary cachexia, muscle wasting secondary to treatment with steroids affect the respiratory muscles whose consequent weakness further exacerbates respiratory failure, prevents weaning from ventilators, and impairs outcome of treatment during acute exacerbations [36].

Elements of MICS have been shown to blunt the responsiveness of anaemia to recombinant human erythropoietin (EPO) in CKD patients [37].

Cachexia and costs to society

The economic costs of cachexia extend much further than the costs of therapeutic diets, nutritional supplements, medications, laboratory tests, and supplies. Staff salaries, service costs, and other indirect medical costs related to the provision of medical care must also be included. Unfortunately, it is difficult to assess accurately the financial costs of cachexia due to paucity of data. The extent of the problem can, however, be highlighted by examining the conditions in which cachexia is prevalent. Estimates for overall yearly costs of chronic diseases such as cancer, CHF, COPD, and ESRF in the United States exceed $286 billion [13,16,38,39]. These include approximately $25 billion in indirect morbidity costs (cost of lost productivity due to illness) and $120 billion in indirect mortality costs (cost of loss of productivity due to premature death).

Less apparent are the costs associated with managing the consequences of involuntary weight loss. Involuntary weight loss is associated with anaemia, postural hypotension, cognitive dysfunction (i.e., confusion and impaired cognition), falls, and hip fractures [40]. Pressure ulcers are another frequent complication seen in patients near the end of the 'cachexia journey' [41]. Currently, billions of dollars are spent each year to treat these complications. For example, the total direct cost of falls is approximately $20 billion and the estimated cost of pressure ulcer management is currently about $7 billion annually [42,43].

Conclusion

In summary, (pre)cachexia (at least defined by weight loss) is present in approximately 2% of the general population. Weight loss in obese patients is associated with increased risk of mortality, which is probably due partly to increased activity of the underlying disease and partly to the development of wasting. Cachexia syndrome (in its more multidimensional form) is probably somewhat less prevalent but associated with greater functional impairment. In all chronic diseases, the development of cachexia carries a significant worsening of an often grave prognosis and has a clear impact on at least the physical function domains of QoL.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 546–547).

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Sarcopenia in an Overweight or Obese Patient Is an Adverse Prognostic Factor in Pancreatic Cancer

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Abstract

Purpose: The average weight-losing pancreatic cancer patient undergoing palliative therapy is frequently overweight rather than underweight, and this can confound conventional measures used for risk stratification. The aim of this study was to evaluate if weight and body composition, specifically sarcopenia, assessed from diagnostic computed tomography (CT) scans, is of prognostic value in patients with pancreatic cancer. The nature and extent of tissue loss over subsequent months was also evaluated.

Experimental Design: A total of 111 patients entering a palliative therapy program, who had CT images and had undergone nutritional screening, were studied. In patients for whom follow-up scans were available ($n = 44$), longitudinal changes in body composition were studied at a mean of 230 ± 62 and 95 ± 60 days prior to demise.

Results: Sixty-two patients (55.9%) were sarcopenic, 44 (39.6%) were overweight/obese, and 18 (16.2%) were both. Age ≥59 years (hazard ratio, 1.71; 95% confidence interval, 1.10-2.66; $P = 0.018$), and overweight/obese sarcopenia (hazard ratio, 2.07; 95% confidence interval, 1.23-3.50; $P = 0.006$) were identified as independent predictors of survival on multivariate analysis. Longitudinal analysis revealed that total fat-free mass index decreased from 15.5 ± 2.5 kg/m^2 to 14.5 ± 2.0 kg/m^2 ($P = 0.002$), and total fat mass index decreased from 7.5 ± 2.0kg/m^2 to 6.0 ± 1.5kg/m^2 ($P < 0.0001$) over 135 days.

Conclusions: Sarcopenia in overweight/obese patients with advanced pancreatic cancer is an occult condition but can be identified using CT scans. This condition is an independent adverse prognostic indicator that should be considered for stratification of patients' entering clinical trials, systemic therapy, or support care programs. (Clin Cancer Res 2009;15(22):6973–9)

Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries (1). At the time of diagnosis, tumor resection with curative intent is only possible in 10% to 15% of subjects (2, 3), leaving a large population with poor prognosis and limited therapeutic options. Overall, the 5-year survival rate is only about 4% (4).

One of the most distressing features of pancreatic cancer is marked and progressive weight loss. Cachexia occurs in up to 80% of deaths in patients with advanced pancreatic cancer (5). Cachexia has been shown to worsen prognosis and has also been associated with impairment of physical function, increased psychological distress, and low quality of life (6, 7). Patients with pancreatic cancer often report a decreased dietary intake and many symptoms such as anorexia, early satiety, anxiety, depression, pain, and nausea (8).

Due to the epidemic of obesity in Western society, a substantial proportion of oncology patients at the start of palliative therapy now have a body mass index (BMI) in the overweight range (9), and this can confound conventional measures used for risk stratification. Indeed recent studies have reported that obesity (i.e., BMI ≥30 kg/m^2) in the presence of sarcopenia is predictive of morbidity and mortality in both malignant and nonmalignant disease (10, 11). The development of novel methods of image analysis enabling routine derivation of body composition data from diagnostic computed tomography (CT) scans (and in particular the estimation of skeletal muscle mass) provides an opportunity to assess if measures of body composition have any prognostic value in patients with pancreatic cancer. The present study focused on sarcopenia specifically, both in the presence or absence of an elevated BMI.

When considering the significance of sarcopenia in a given population, it is important to know the likely longitudinal

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Translational Relevance

In the present study, patients had a mean body mass index of 23.9 with 40% of individuals being in the overweight/obese range. However, underneath this mantle of adipose tissue the previously noted tendency to muscle wasting continues. The use of computed tomography (CT) images in the present study identified that 56% of patients had sarcopenia at the time of presentation. This study also shows that sarcopenia in overweight or obese pancreatic cancer patients is an independent determinant of poor prognosis. Routine diagnostic CT scans are a good resource for detailed nutrition/metabolic assessment of patients and the identification of overweight/obese sarcopenia. The presence of overweight/obese sarcopenia should be considered in the stratification of patients' entering clinical trials, systemic therapy, or support care programs.

pattern of wasting and how this may be altered by concomitant systemic oncologic therapy. The nutritional and metabolic status of patients who respond to chemotherapy may improve spontaneously, and a substantial proportion of patients now receive antineoplastic therapy even in the last weeks of life. Another aim of the present study was to assess time course changes in regional body fat and lean tissue compartments by analyzing CT images in a subset of pancreatic cancer patients being managed within a regional palliative therapy program.

Materials and Methods

Patients. All patients referred to the regional cancer center in Edmonton, Alberta, Canada from January 2004 to October 2008 were considered for the study. Patients with a primary diagnosis of pancreatic cancer entering a palliative program who had an abdominal CT scan within 60 d of initial assessment were selected for the study (n = 111). Patients with ampullary carcinoma, cholangiocarcinoma, or neuroendocrine tumors were excluded. Coding of the primary cancer by its site and morphology, clinical information, and demographic information were obtained from the Alberta Cancer Registry for every patient in the cohort. The Alberta Cancer Registry is a computerized database of all cancer cases in the region (population 1.8 million). Patient-reported height, weight, and weight history were collected during this visit by use of the Patient-Generated Subjective Global Assessment (12). Height and weight data were subsequently used to compute a common anthropometric descriptor, BMI (kg/m^2). Height and weight recorded by hospital staff on the same date were used for verification where available, and classification of patients' BMI with the use of patient-reporting was found to be accurate. Patient-reported height, weight, and weight history have been shown to be reliable (13). Stage of disease was based on the American Joint Committee on Cancer stage groupings I, II, III, and IV.

From the initial cohort of 111 patients, 44 patients were further identified who: (*a*) had had at least one further follow-up CT scan, and (*b*) had a documented duration of survival for inclusion into the study of longitudinal changes of body composition.

CT image analysis. CT scans used for analysis were done solely for routine cancer care. Two consecutive transverse CT images extending from the third lumbar vertebrae (L3) in the inferior direction were assessed for each scan date and then averaged, the foremost image being the one in which both transverse processes were first clearly visible. Images were analyzed with SliceOmatic V4.3 software (Tomovision), which enables specific tissue demarcation using Hounsfield unit (HU) thresholds. Skeletal muscle was identified and quantified by HU thresholds of -29 to +150 (14). The muscles in the L3 region contain *psoas, erector spinae, quadratus lumborum, transversus abdominus,* external and internal obliques, and *rectus abdominus.* The following HU thresholds were used for adipose tissues: -190 to -30 for s.c. and i.m. adipose (15), and -150 to -50 for visceral adipose (16). Tissue boundaries were manually corrected as needed. Cross-sectional areas (cm^2) were computed automatically by summing tissue pixels and multiplying by pixel surface area. All CT images were analyzed by a single trained observer. Cross-sectional area for muscle and adipose tissue was normalized for stature (cm^2/m^2) and reported.

Routine diagnostic CT scans usually only evaluate the chest, abdomen, and pelvis, and therefore only partial images are available to determine skeletal muscle mass. Estimates of whole body stores were generated from the raw data (cm^2) using the following regression equations by

Table 1. Overall patient demographics, nutritional variables, and body composition at the time of assessment

	No. of patients (n = 111)
Age (y)*	64.4 ± 9.3
Sex	
Male	52 (46.8)
Female	59 (53.2)
Tumor site	
Head of pancreas	57 (61.3)†
Body of pancreas	18
Tail of pancreas	4
Pancreatic duct	1
Neck of pancreas	2
Overlapping lesion	11
Not recorded	18
Histology	
Adenocarcinoma	84 (75.7)
Unknown	27
Stage	
II	1
III	7
IV	103 (92.8)
BMI (kg/m^2)*	23.9 ± 4.9
Underweight (BMI <18.5 kg/m^2)	11 (9.9)
Normal (BMI 18.5-24.9 kg/m^2)	56 (50.5)
Overweight/obese (BMI ≥25 kg/m^2)	44 (39.6)
Percentage weight loss (in preceding 6 mo)*	12.14 ± 6.35
Lumbar total muscle cross-sectional area (cm^2)*	126.0 ± 30.7
Lumbar total adipose tissue cross-sectional area (cm^2)*	243.7 ± 162.3
Lumbar skeletal muscle index (cm^2/m^2)*	43.8 ± 7.9
Lumbar adipose tissue index (cm^2/m^2)*	86.1 ± 57.4
Estimated total fat-free mass (kg)*	43.9 ± 9.2
Estimated total fat mass (kg)*	21.2 ± 6.9
Sarcopenic	62 (55.9)
Overweight/obese and sarcopenic	18 (16.2)
Status	
Dead	101 (91.0)
Alive	10

NOTE: Values are number of patients with percentages in parentheses unless indicated otherwise.
*Values are mean ± SD.
†Unknown tumor site was excluded from calculation of overall percentage.

Mourtzakis et al. (17), which show a close correlation between muscle and fat areas in CT images at the third lumbar vertebrae and whole body compartments of fat-free mass (FFM) and fat mass (FM) respectively.

Total body fat-free mass (FFM) (kg) = 0.3 × [skeletal muscle at L3 (cm^2)] + 6.06 ($r = 0.94$)

Total body fat mass (FM) (kg) = 0.042 × [total adipose tissue at L3 (cm^2)] + 11.2 ($r = 0.88$)

The respective indexes for FFM and FM (kg/m^2) were also reported.

CT dates were expressed in terms of the number of days to death. Any change in tissue area was expressed as either an absolute change (cm^2) or as a percentage change per 100 days. This provided a standardized unit and allowed for comparison across different intervals.

Cutoffs for sarcopenia were based on a CT-based sarcopenic obesity study of cancer patients by Prado et al. (i.e., L3 skeletal muscle index: ≤38.5 cm^2/m^2 for women and ≤52.4 cm^2/m^2 for men; ref. 10).

Statistical analysis. Data are presented as mean ± SD unless otherwise stated. Survival was determined from the time of initial assessment until death or until the censor date of January 5, 2009.

Univariate and multivariate survival analyses and calculation of hazard ratios were done using a Cox regression model. Owing to the large number of covariates examined, only those that were significant on univariate analysis were included in multivariate analysis. Receiver-operator characteristic curves were used to select cutoff values for continuous variables. Values with the best combination of sensitivity and specificity were chosen. A backward stepwise procedure was done to derive a final model of the variables that had a significant relationship with survival. To remove a variable from the model, the corresponding P value had to be >0.05.

Comparisons between groups of patients were assessed using one-way ANOVA or Pearson's χ^2 test. Survival curves were constructed using the Kaplain-Meier technique. Log-rank test was used to compare survival between groups of patients. Comparison of data at different time points for body composition analysis was done using the paired t-test. P values <0.05 were regarded as statistically significant. Statistical analysis was done using SPSS 15.0 statistical package (SPSS Inc.).

Results

Details of the 111 pancreatic cancer patients identified at the time of referral to the cancer center are shown (Table 1). About two thirds of the patients had tumors at the head of the pancreas. Approximately 75% of patients had biopsy-proven adenocarcinoma. At the time of censoring, 101 patients (91.0%) had died. Overall median survival was 130 days [interquartile (IQ) range, 71-302 days]. Percentage weight loss in the preceding six months was 12 ± 6%, with 89 patients (80.2%) losing >5% of their normal body weight. BMI at the time of assessment was 23.9 ± 4.9 kg/m^2, and 44 patients (39.6%) were overweight or frankly obese (BMI ≥25 kg/m^2). Body composition parameters of patients are also reported in Table 1. Sixty-two patients (55.9%) were sarcopenic at this point; 18 patients were overweight/obese (BMI ≥25 kg/m^2) and sarcopenic. The prevalence of sarcopenia within the various BMI categories is presented in Fig. 1.

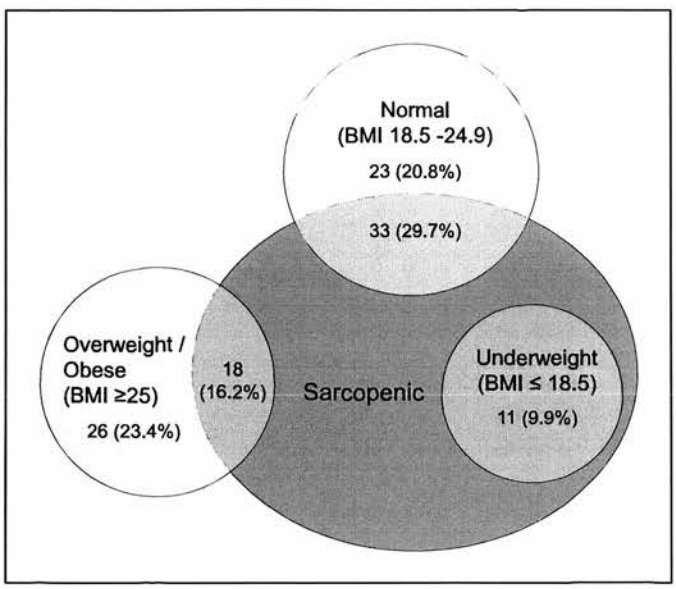

Fig. 1. Venn diagram of BMI classes and sarcopenic patients.

Patients were then divided into four groups: neither sarcopenic nor overweight/obese, overweight/obese, sarcopenic, and both sarcopenic and overweight/obese (Table 2). There were no significant differences in age, sex, tumor site, histology, stage of disease, and weight loss among the groups.

On univariate analysis, age and overweight/obese sarcopenia were associated with outcome for the patient group. Overweight/obesity (BMI ≥25 kg/m^2) alone as well as sarcopenia on its own failed to reach statistical significance for patient outcome (Table 3). In contrast, median survival for patients who were both overweight/obese and sarcopenic was 55 days (IQ range, 43-207 days) compared with 148 days (IQ range, 80-369 days) for the rest of the patient cohort without overweight/obese sarcopenia (log-rank test, $P = 0.003$; Fig. 2). Using receiver-operator characteristic curves, the cutoff value with the best discriminatory value for age was ≥59 years.

On multivariate analysis, age ≥59 years (hazard ratio, 1.71; 95% confidence interval, 1.10-2.66; $P = 0.018$) and overweight/obese sarcopenia (hazard ratio, 2.07; 95% confidence interval, 1.23-3.50; $P = 0.006$) retained independent prognostic value (Table 3).

Longitudinal analysis of body composition. A subset of 44 patients underwent repeated CT scans as part of their medical management and was therefore available for study of longitudinal changes in body composition. This cohort of patients had a significantly longer survival compared with the entire group (median survival, 189 days versus 130 days; $P = 0.019$, log-rank test). Seventy-one percent of patients with follow-up CT scans received active treatment compared with just 28% of patients who had no follow-up CT scans ($P < 0.0001$, χ^2 test). Patients had their first CT scan at a mean of 230 ± 62 days before death. The second CT scan was done at a mean of 95 ± 60 days before death.

The changes in cross-sectional area of skeletal muscle and adipose tissue between the two scans are presented in Table 4. Patients displayed a significant loss of both skeletal muscle and adipose tissue. Overall, 32 patients (72.7%) lost skeletal muscle and all but one patient (97.6%) lost adipose tissue. The measurements for cross-sectional area were used to estimate whole body FFM and whole body FM using regression equations and then normalized for height (kg/m^2; Table 4).

Twenty patients (45.5%) were sarcopenic at the time of the first CT, with an estimated FFM index of 15.5 ± 2.5 kg/m^2. By the time of the second CT, 27 patients (61.4%) were sarcopenic and FFM had decreased to 14.5 ± 2.0kg/m^2. Estimated FM index decreased from 7.5 ± 2.0 kg/m^2 (1st CT) to 6.0 ± 1.5 kg/m^2 (2nd CT).

Table 2. Comparison of demographic characteristics and body composition of patients who were neither overweight nor sarcopenic, sarcopenic alone, overweight alone, and both overweight and sarcopenic

	BMI <25 kg/m²		BMI ≥25 kg/m²		*P*
	Not overweight, not sarcopenic (*n* = 23; 21%)	Not overweight, sarcopenic (*n* = 42; 38%)	Overweight/Obese (*n* = 28; 25%)	Overweight/Obese and sarcopenic (*n* = 18; 16%)	
Age (y)					
Mean ± SD	60.7 ± 7.5	65.8 ± 10.2	64.3 ± 9.0	66.0 ± 9.3	0.169*
Sex, *n* (%)					
Male	8 (34.8)	20 (47.6)	11 (39.3)	13 (72.2)	0.084†
Female	15 (65.2)	22 (52.4)	17 (60.7)	5 (27.8)	
Tumor site, *n* (%)					
Head	8 (34.8)	26 (61.9)	14 (50.0)	9 (50.0)	0.088†
Body	9 (39.1)	3 (7.1)	4 (14.3)	2 (11.1)	
Overlapping lesion	3 (13.0)	3 (7.1)	2 (7.1)	3 (16.7)	
Histology, *n* (%)					
Adenocarcinoma	18 (78.3)	28 (66.7)	22 (78.6)	16 (88.9)	0.287†
Stage, *n* (%)					
IV	20 (87.0)	37 (88.1)	28 (100)	18 (100)	0.149†
BMI (kg/m²)					
Mean ± SD	22.2 ± 1.9	20.5 ± 2.8	27.6 ± 2.7	28.5 ± 6.3	<0.0001*
% Weight loss					
Mean ± SD	12.83 ± 5.80	13.08 ± 6.45	11.36 ± 6.72	10.45 ± 6.24	0.435*
Lumbar total muscle cross-sectional area (cm²)					
Mean ± SD	129.5 ± 31.8	115.0 ± 29.2	138.5 ± 29.6	128.0 ± 27.6	0.014*
Lumbar total adipose tissue cross-sectional area (cm²)					
Mean ± SD	185.0 ± 137.7	168.8 ± 130.6	344.2 ± 148.0	348.6 ± 155.1	<0.0001*
Lumbar skeletal muscle index (cm²/m²)					
Mean ± SD	46.2 ± 7.1	39.3 ± 6.2	49.4 ± 7.3	42.7 ± 6.6	<0.0001*
Lumbar adipose tissue index (cm²/m²)					
Mean ± SD	68.4 ± 52.3	57.6 ± 42.0	125.6 ± 55.7	117.1 ± 50.7	<0.0001*
Estimated total fat-free mass (kg)					
Mean ± SD	44.9 ± 9.5	40.6 ± 8.8	47.6 ± 8.9	44.5 ± 8.3	0.014*
Estimated total fat mass (kg)					
Mean ± SD	19.0 ± 5.8	18.3 ± 5.5	25.7 ± 6.2	24.2 ± 7.7	<0.0001*

*One-way ANOVA.
†Pearson's χ^2 test.

A distinct distribution for muscle and adipose tissue changes over time is more clearly revealed by analysis of population tertiles (Fig. 3). The overall change of skeletal muscle was -3.1 ± 12.0%/100 days. However, the 1st tertile gained a small amount of muscle tissue (7.9 ± 14.4%/100 days) whereas the 3rd tertile lost muscle at a rate of -12.7 ± 5.2%/100 days. In comparison, adipose tissue was lost across all three tertiles and the overall change of adipose tissue was -40.4 ± 25.4%/100 days (P <

Table 3. Hazard ratio for risk of death associated with clinical variables and body composition in pancreatic cancer patients (n = 111)

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P**	Hazard ratio	95% CI	*P*†
Age	1.025	1.002-1.048	0.03			
≥59 y				1.708	1.097-2.660	0.018
Sex	0.793	0.534-1.178	0.25			
Tumor site	1.031	0.977-1.087	0.268			
Histology	0.791	0.499-1.254	0.32			
Stage	1.829	0.910-3.677	0.09			
Overweight/obese vs. normal/under weight	1.454	0.969-2.181	0.071			
Percentage weight loss	0.991	0.959-1.025	0.599			
Lumbar skeletal muscle index (cm²/m²)	1.001	0.976-1.026	0.964			
Lumbar adipose tissue index (cm²/m²)	1.003	0.999-1.007	0.153			
Sarcopenia vs. no sarcopenia	1.284	0.863-1.910	0.217			
Sarcopenia plus overweight/obese vs. other patients	2.177	1.292-3.670	0.003	2.071	1.227-3.496	0.006

Abbreviations: 95% CI, 95% confidence interval.
*Cox univariate analysis.
†Backward conditional method of Cox proportional hazards model.

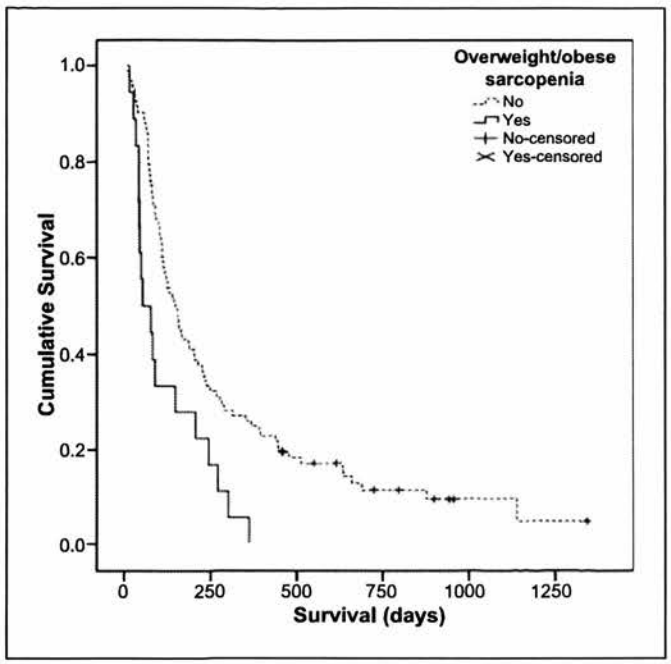

Fig. 2. Survival curves of patients with overweight/obese sarcopenia and patients without overweight/obese sarcopenia.

0.0001, paired *t*-test). The proportion of patients receiving chemotherapy was not significantly different across all three tertiles of muscle ($P = 0.372$, χ^2 test) or adipose tissue loss ($P = 0.804$, χ^2 test). Moreover, the changes in muscle ($P = 0.113$, Student's *t*-test) and fat mass ($P = 0.862$, Student's *t*-test) were not significantly different between those who did or did not receive chemotherapy. There was also no significant difference in survival across all three tertiles for both muscle loss ($P = 0.142$, log-rank test) and adipose tissue loss ($P = 0.542$, log-rank test).

Discussion

Patients with pancreatic cancer have long been associated with the most severe forms of cachexia. In a similar study undertaken more than 10 years ago, the present authors documented median BMI at diagnosis to be 20.7, and this fell to 17.7 near to the time of death (18). Average weight loss over this time increased from 15% to 25%. Loss of muscle and fat to levels consistent with significant undernutrition increased from 30% to 70% and from 65% to 90%, respectively. This was consistent with the conventional view of cancer cachexia (i.e., marked weight loss, severe muscle wasting and gross loss of s.c. fat; ref. 19). In the present study, patients had a mean BMI of 23.9 with 40% of individuals being in the overweight/obese range. Thus, the average physiognomy seems to have changed with patients showing large energy reserves (fat) at the time of presentation with advanced disease. However, underneath this mantle of adipose tissue the previously noted tendency to muscle wasting continued. The use of CT images in the present study identified that 56% of patients had sarcopenia at the time of presentation (Table 1) and the tendency to muscle loss continued in at least a proportion of patients (Fig. 3).

A BMI <18.5kg/m^2 is considered by many authorities to represent an individual at serious risk of undernutrition (20). In the present study, only 10% of individuals at baseline fulfilled this criterion. Given the prevalence of overweight/obesity (40%) it would seem unlikely that even in the presence of ongoing weight loss, the majority would reach this boundary at or near the time of death. However, BMI has clear limitations, and more detailed evaluation of body composition clearly revealed wasting of the lean tissues, with a majority of patients below or well below benchmark levels of muscularity known to be associated with mortality and functional disability (21). The estimated lean body mass of patients classified as sarcopenic was within the range described for a variety of wasted/emaciated patient populations with and without malignant disease (10, 22). In the current literature it is becoming increasingly evident that concurrent sarcopenia and high fat mass is a worst case scenario (10, 11, 23, 24), and this was clearly apparent in our study group (albeit small), in which sarcopenic overweight/obese patients had the worst prognosis overall, even compared with patients who were sarcopenic and had a lower body weight.

There is ongoing controversy as to what the best definition for sarcopenia is. Mourtzakis et al. have previously shown that CT cross-sectional area at L3 is strongly related to appendicular skeletal mass, measured by dual-energy X-ray densitometry, used commonly in the definition of sarcopenia (17). Subsequent derived cutoffs for sarcopenia, based on CT, used in this study are in fact close to that described by Baumgartner et al.

Table 4. Change in body composition over time in pancreatic cancer patients ($n = 44$)

	First CT scan	Second CT scan	Δ	*P**
Time to death (d)	230 ± 62	95 ± 60	135 ± 57	
Skeletal muscle (cm^2)	126.5 ± 31.1	119.6 ± 28.6	-7.0 ± 13.6	0.002
Adipose tissue (cm^2)				
Visceral adipose tissue	91.2 ± 69.9	45.4 ± 47.0	-45.8 ± 47.5	<0.0001
Intramuscular and subcutaneous adipose tissue	148.5 ± 85.8	87.0 ± 66.4	-61.5 ± 53.9	<0.0001
Total	236.6 ± 145.5	128.6 ± 102.6	-108.0 ± 89.1	<0.0001
Estimated whole body FFM (kg)	44.0 ± 9.3	41.9 ± 8.6	-2.1 ± 4.1	0.002
Estimated whole body adipose tissue (FM) (kg)	21.1 ± 6.1	16.6 ± 4.3	-4.5 ± 3.7	<0.0001
Estimated FFM index (kg/m^2)	15.5 ± 2.5	14.5 ± 2.0	-1.0 ± 1.5	0.002
Estimated FM index (kg/m^2)	7.5 ± 2.0	6.0 ± 1.5	-1.5 ± 1.5	<0.0001

NOTE: Values are mean ± SD.
*Paired *t*-test.

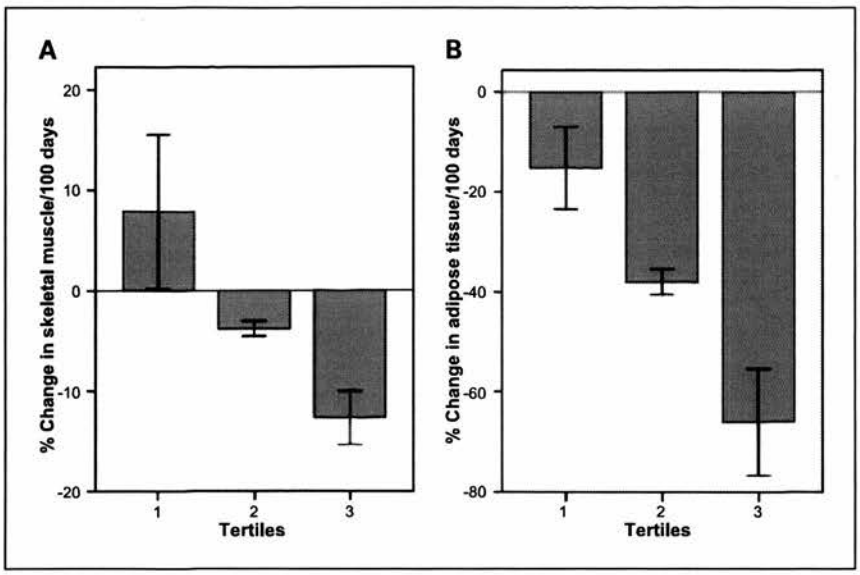

Fig. 3. Intensity of changes in body composition presented by tertiles. *A*, skeletal muscle. *B*, adipose tissue.

(ref. 22; i.e., appendicular skeletal mass >2 SDs below a young healthy adult population). Equally, the optimal regression for conversion of CT image data to conventional units of whole-body composition measures has yet to be resolved in large population studies. In the present study, previously reported regression equations determined from a heterogeneous group of cancer patients were used (17).

Sarcopenia alone had no discernable effect on mortality, yet being overweight/obese and sarcopenic was associated with decreased survival duration. However, a variable can only serve a predictive function where it varies in the relevant population. It should be noted that all patients with a BMI <18.5 were sarcopenic, and the majority were sarcopenic in the BMI range of 18.5-24.9. Thus, one explanation for the finding that sarcopenia was not predictive for the overall population, but was predictive for those with obesity, would lie in the differential frequency distribution of sarcopenia in the different BMI categories. Evaluation of this question in a much larger patient population would help resolve this issue. There has also been some evidence that body composition changes in advanced cancer may have different impacts on survival in males and females (25). However, the present study was not powered sufficiently to analyze the differences in gender due to the small sample sizes. It would be interesting to evaluate this in a larger population.

In the present study, patients entering the palliative phase of their pancreatic cancer management had a remarkably high prevalence of sarcopenia compared with reports in the literature for healthy elderly in a similar age bracket (22), thereby suggesting that substantial muscle wasting had occurred prior to initial evaluation of these patients. The pre-existing nature of this muscle loss makes it difficult to comment as to its mechanisms, except perhaps to suggest that it may be driven by the primary malignancy, by weight loss, and/or by comorbid conditions that include obesity, insulin resistance, various types of organ failure, and low levels of physical activity. The muscle loss that occurred thereafter, which was characterized during the 135-days scan-scan interval, may be driven by disease progression, increased metabolism, and inflammation (26, 27), and by negative energy balance that might be inferred from the loss of fat mass (i.e., 4.54 kg × ~9,000 kcal/kg = 40,840 kcal) in 135 days.

The mechanism that links sarcopenic overweight/obesity with accelerated demise is not known. Muscle wasting is a known complication associated with insulin resistance found commonly in obesity (28). Adipose tissue synthesizes and secretes circulating hormones and "adipokines" that act as systemic inflammatory mediators and signals of nutritional status (29). These adipocyte factors, such as tumor necrosis factor-α and interleukin-6, are thought to play a major role in the induction of insulin resistance in skeletal muscle leading to an increase in muscle protein loss. The main mediators thought to be involved in this process are inhibitor κB kinase and its downstream effector NF-κB (30). However, not all patients who are overweight/obese have sarcopenia. It may be that cancer-related factors stimulate the initial loss of muscle, and being overweight/obese perpetuates and/or enhances muscle loss/loss of muscle function leading to poorer survival. The observation that overweight/obesity may be associated with better survival in patients with weight-losing cardiac failure (31) may seem to contradict the present observations in cancer patients. However, the studies in patients with cardiac failure have not been stratified for body composition (specifically sarcopenia) and may represent a disease-specific phenomenon.

Current published results on body composition changes in cancer are varied. Some studies confirm a decline in lean body mass (32, 33), whereas others emphasize a loss of body fat (34, 35). There are also reports that suggest a proportional loss of lean tissue and fat leading to an unchanged body composition (36, 37). In the present study, roughly half of the patients were found to be already sarcopenic at the time of assessment. Subsequently, a much greater rate of fat loss was noted as compared with muscle loss (see longitudinal study). In fact, some patients were able to maintain or even gain muscle mass. The patients that gained muscle could have been positive responders to chemotherapy. However, in patients with pancreatic cancer, the use of CT scans to determine response to chemotherapy is complicated by factors such as peritumoral inflammation, therefore duration of survival was regarded as a more robust measure (accepting the small numbers involved in this study). In the present study the survival of patients was not significantly different across all three tertiles of loss of muscle

mass. Thus, response to chemotherapy as the main reason for patients to gain muscle mass cannot be confirmed. An alternative hypothesis would be that a proportion of patients were able to activate compensatory mechanisms aimed at conserving muscle. There is increasing evidence that gene polymorphisms are related to cancer cachexia susceptibility (38, 39).

Systemic inflammation is known to be a key mediator in cachexia (40), and has been associated with poor prognosis in previous studies on pancreatic cancer (41, 42). The present study lacks measures of systemic inflammation such as C-reactive protein, which may be significant when assessing factors influencing survival in pancreatic cancer. Nevertheless, this study has shown that advanced pancreatic cancer patients who are both overweight/obese and sarcopenic can be identified using diagnostic CT scans. Due to the very short median survival of such patients (55 days) overweight/obese sarcopenia should be taken into consideration when planning whether systemic anticancer therapy is appropriate. Moreover, overweight/obese sarcopenia should be considered for stratification of patient's entering clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Genome Medicine

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Using transcriptomics to identify and validate novel biomarkers of human skeletal muscle cancer cachexia

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Abstract

Background: Cancer cachexia is a multi-organ tissue wasting syndrome that contributes to morbidity and mortality in many cancer patients. Skeletal muscle loss represents an established key feature yet there is no molecular understanding of the disease process. In fact, the postulated molecular regulators of cancer cachexia originate largely from pre-clinical models and it is unclear how these translate to the clinical environment.

Methods: *Rectus abdominis* muscle biopsies were obtained from 65 upper gastrointestinal (UGI) cancer patients during open surgery and RNA profiling was performed on a subset of this cohort (n = 21) using the Affymetrix U133+2 platform. Quantitative analysis revealed a gene signature, which underwent technical validation and independent confirmation in a separate clinical cohort.

Results: Quantitative significance analysis of microarrays produced an 83-gene signature that was able to identify patients with greater than 5% weight loss, while this molecular profile was unrelated to markers of systemic inflammation. Selected genes correlating with weight loss were validated using quantitative real-time PCR and independently studied as general cachexia biomarkers in diaphragm and *vastus lateralis* from a second cohort (n = 13; UGI cancer patients). CaMKIIβ correlated positively with weight loss in all muscle groups and CaMKII protein levels were elevated in *rectus abdominis*. TIE1 was also positively associated with weight loss in both *rectus abdominis* and *vastus lateralis* muscle groups while other biomarkers demonstrated tissue-specific expression patterns. Candidates selected from the pre-clinical literature, including FOXO protein and ubiquitin E3 ligases, were not related to weight loss in this human clinical study. Furthermore, promoter analysis identified that the 83 weight loss-associated genes had fewer FOXO binding sites than expected by chance.

Conclusion: We were able to discover and validate new molecular biomarkers of human cancer cachexia. The exercise activated genes *CaMKIIβ* and *TIE1* related positively to weight-loss across muscle groups, indicating that this cachexia signature is not simply due to patient inactivity. Indeed, excessive CaMKIIβ activation is a potential mechanism for reduced muscle protein synthesis. Our genomics analysis also supports the view that the available preclinical models do not accurately reflect the molecular characteristics of human muscle from cancer cachexia patients.

Background

Cancer cachexia is a syndrome associated with malignant tumor disease defined by weight loss, asthenia and anorexia. Up to half of all cancer patients are affected, leading to increased morbidity and poor prognosis [1] with perhaps 20% of cancer deaths being related to cachexia rather than direct tumor effects [2]. Cachectic patients suffer loss of both muscle mass and adipose tissue (with comparative sparing of visceral protein) and this tissue loss appears resistant to nutritional support [3,4]. A PubMed analysis indicates that almost one-third of documents discussing cancer cachexia are review articles, highlighting the need for more primary investigations to

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shed light on the detailed mechanisms that produce the syndrome in patients. Furthermore, most molecular hypotheses have been generated using pre-clinical models or reflect biochemical concepts [5] and there has been little progress in relating these potential mechanisms to changes observed in the patient.

Muscle mass is maintained by physical activity, reflecting a balance between protein synthesis and degradation. Intracellular protein breakdown involves the ubiquitin proteasome pathway (UPP) and the autophagy (lysosomal), caspase, cathepsin and the calcium-dependent calpain pathways. The individual prominence of each of these pathways in muscle wasting conditions is still debated. Many of the molecular signaling pathways that are postulated to contribute to muscle atrophy in pre-clinical models mediate their effects through activation of the UPP [6]. Identification of two muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1, in a large number of animal models of atrophy [7,8] has been used to provide an argument for a major contribution of the UPP in muscle wasting, such that these genes are now measured as surrogate indicators of UPP activation. It should be kept in mind that active tissue remodeling, even with net protein accretion, may well rely partly on the protein degradation pathways and, as such, they may not represent logical surrogates for commenting on net protein degradation.

In humans, reduced levels of phosphorylated (inactive) FOXO3a have been observed in the skeletal muscle of cachectic compared with non-cachectic cancer patients, but an unexplained twofold reduction in the amount of FOXO1 and FOXO3a was also observed [9], making the data challenging to interpret. FOXO3 also appears to induce expression of autophagy-related genes [10-13], suggesting a link between the lysosomal and proteasomal systems. However, there is also evidence that the UPP is first activated with increasing weight loss then declines as the disease severity progresses [14]. This suggests that UPP is a marker of protein turn-over rather than wasting *per se* (with turn-over increasing as the muscle weakens, but only while the patient continues to be ambulatory) or that UPP proteins are not reliable biomarkers. Furthermore, recent data indicates a dissociation between protein dynamics *in vivo* and activation or expression of the UPP-related signaling molecules in human skeletal muscle [15]. Overall, it is not clear what regulates muscle mass *in vivo* nor is it clear to what extent protein degradation contributes over inhibition of protein synthesis [15,16]. Given the paucity of data derived from cancer cachexia patients, including study of the UPP and autophagy systems, we sought to carry out both targeted and global molecular profiling in the skeletal muscle of cancer patients and relate our findings to clinical status.

Methods

Men and non-pregnant women over 18 years of age were recruited to the study from two separate centers. Written informed consent was obtained from all subjects and ethical approval received from Lothian Research Ethics Committee (UK) and the Regional Ethics Committee in Stockholm (Sweden). Participating patients had a diagnosis of upper gastrointestinal cancer (esophageal, gastric, pancreatic) and were undergoing surgery with the intent of resection of the primary tumor. A small number of weight stable (WS) patients undergoing surgery for benign, non-inflammatory conditions (n = 7) were also included in the analysis. In center 1 (Edinburgh, UK) a fasting venous blood sample was taken and serum C-reactive protein measured as a marker of systemic inflammation (SI). Body mass index (BMI) and mid-arm muscle circumference were calculated. Clinical details and degree of weight loss from self-reported pre-illness stable weight were recorded. A weight loss ≥5% identified weight-losing (WL) cancer patients as opposed to weight stable (WS) individuals. A serum C-reactive protein ≥5 mg/l was used to define the presence of SI. For patients from center 2 (Stockholm, Sweden) weight and self-reported change in weight over time were recorded. Rate of weight loss was therefore used in these subjects. Due to the small number of controls (otherwise considered as non-cancer patients but with other co-morbidities) and the lack of detailed knowledge of their physical capacity, the primary analysis strategy was chosen to generate molecular changes that varied with the severity of weight loss in patients in center 1 and validate such changes in the independent cohort from center 2 using more than one muscle type. This strategy was devised to provide a stringent test of the molecular changes, as the conclusions are based on a relatively large number of patients with otherwise similar clinical characteristics.

All biopsies were taken at the start of open abdominal surgery. In center 1, the edge of the *rectus abdominis* was exposed and a 1-cm^3 specimen removed using sharp dissection. The biopsy was snap frozen in liquid nitrogen and stored at -80°C until further analysis. In center 2, *vastus lateralis* muscle biopsies were taken with a Bergstrom needle and diaphragm biopsies were obtained by sharp dissection when possible. Both samples were snap frozen and stored at -80°C for further analysis. Approximately 20 mg of frozen tissue was homogenized in 0.5 ml of lysis buffer (Triton - X100 (1%), NaCl (150 mM), Tris-HCl (50 mM), EDTA (1 mM), PMSF (1 mM), protease inhibitors (Roche Diagnostics, Burgess Hill, UK); 1 tablet per 10 ml), water to 10 ml) using a Powergen 125 (Fisher Scientific, Loughbourgh, UK)) electric homogenizer. Samples were left on ice for 15 minutes prior to centrifuging at 13,000 rpm for 15 minutes. The supernatant was removed, and protein concentration was

determined by comparing equal volumes of sample solution to known standards using the Lowry method. Samples were then stored at -80°C.

Approximately 20 mg of muscle was re-suspended in 180 μl of low salt lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, protease inhibitors (Roche Diagnostics; 1 tablet per 10 ml)) and ground using a handheld homogenizer. Samples were incubated on ice for 5 minutes before two cycles of freeze-thaw lysis. After a brief vortex, samples were centrifuged at 4,000 rpm for 3 minutes. The supernatant was removed and the pellet (containing the nuclei) re-suspended in 40 μl high salt extraction buffer (20 mM HEPES, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, protease inhibitors (Roche Diagnostics; 1 tablet per 10 ml)). Samples were incubated on ice for 30 minutes with gentle mixing of the tubes every 5 to 10 minutes. Samples were centrifuged at 4,000 rpm for 5 minutes at 4°C. An aliquot of supernatant (containing the nuclear proteins) was stored at -80°C.

Protein from each sample (20 μg) was added to 3 μl of 4 × loading buffer solution (0.5 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.05% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 3 minutes. Proteins were resolved using SDS-PAGE at 160V for 45 minutes. Proteins were transferred to a nitrocellulose membrane (80 mA for 1 hour) using semi-dry transfer (Biorad, Hemel Hempstead, UK). Membranes were blocked with either 3% bovine serum albumen/tris-buffered saline (TBS) with Tween 20 (TBST; 0.05% Tween) overnight at 4°C or with 5% milk/TBST for 1 hour at room temperature. Incubation with primary antibody (1:1,000) was carried out in either 3% bovine serum albumen/TBST or 0.5% milk/TBST solution at room temperature for 2 hours or overnight at 4°C. Membranes were washed with TBST and primary antibody binding detected using horseradish-peroxidase conjugated secondary antibodies (1:2,000 to 1:5,000; anti-mouse, anti-rabbit (Upstate, Dundee, UK)). Specific signal was detected using ECL reagent (GE Healthcare, Little Chalfont, UK) and exposure on photographic film (Kodak). Films were scanned and densitometry values estimated using ImageJ (NIH) software. The primary antibodies used in the study were against phos-CaMKII(Thr286), FOXO1 and FOXO3a (New England Biolabs, Hitchin, UK), Lamin A/C (Insight, Wembely, UK), alpha-skeletal actin (Novocaestra, Newcastle, UK) and calcium/calmodulin-dependent protein kinase (CaMK)II (BD Biosciences, Oxford, UK).

Total RNA was extracted from approximately 20 mg of muscle using TRIzol (Invitrogen, Paisley, UK) reagent according to the manufacturer's directions. The RNA pellet was re-suspended in diethylpyrocarbonate-treated water and RNA concentration was determined using a Nanodrop spectrophotometer (LabTech International, Ringmer, UK). RNA quality was assessed using 260/280, 230/260 ratios and the RNA integrity number (RIN) score from the BioAnalyzer 2100 instrument (Agilent Technologies, Stockport, UK). Total RNA (3.5 μg) was reverse transcribed and processed according to the protocol provided by Affymetrix Inc. for the GeneChip Expression 3' Amplification One-Cycle Target Labeling and Control Reagents kit (Affymetrix, High Wycombe, UK). Reverse transcription and second strand cDNA synthesis were followed by *in vitro* transcription and biotinylation. Biotinylated cRNA products were cleaned up using columns (Affymetrix). The quality of the biotinylated cRNA was assessed by Nanodrop (LabTech International, UK) and BioAnalyzer (Agilent Technologies) instruments and the cRNA was then fragmented according to Affymetrix protocols. Samples were hybridized to the HGU-133plus2 GeneChip array (covering approximately 54,000 sequences). The raw data files can be accessed at the Gene Expression Omnibus using the ID [GEO:GSE18832].

For quantitative real time PCR (qRT-PCR), cDNA was prepared using 1 μg RNA, TaqMan reverse transcription reagents (Applied Biosystems, Warrington, UK) and random hexamer primers (Applied Biosystems). Primers were designed to span introns using Primer Express 3.0 software (Applied Biosystems) and constructed by Invitrogen (Paisley, UK); primer sequences are detailed in Table S1 in Additional data file 1. Samples were run on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems) in triplicates of 20 μl per well using SYBR Green PCR Master Mix (Applied Biosystems) as per the manufacturer's instructions. Expression levels were normalized to ribosomal 18S RNA and results examined using the ΔCt method [17]. SPSS (SPSS Inc, Chicago, IL, USA) or GraphPad (GraphPad Software, La Jolla, CA, USA) statistical software was utilized. Student's two tailed *t*-test or one way ANOVA (analysis of variance) was used to compare means between groups. Log transformation was used when appropriate. Mann-Whitney was used for nonparametric analysis. Contingency tables were constructed where relevant and analyzed by Fisher's exact test. Statistical significance was set at $P < 0.05$.

Microarray data were analyzed using the Microarray Suite software (MAS) version 5.0 (Affymetrix). To improve the accuracy of the gene to probe relationship, a custom chip definition file (CDF) [18] was used defining the Affymetrix probes by Ensembl transcript ID. Data were normalized using MAS5 and robust multi-array average [19]. Genes called absent on every array by the MAS5 software were filtered from the data and remaining genes analyzed using the quantitative function in significance analysis of microarrays (SAM) [20] implemented in the Bioconductor suite [21]. Percentage weight

Microarray analysis: novel genes associated with weight loss in cancer (centre 1)

The microarray study was undertaken on *rectus abdominis* muscle from a subgroup of center 1 patients (Table 2). Hierarchical and k-means clustering were undertaken with normalized data, using a gene list where those with a low standard deviation were removed. No pattern emerged from this analysis. Using the probe-sets that detect atrogenes (genes reproducibly detected in pre-clinical models of cachexia), which we have previously demonstrated reliably change in human skeletal muscle sepsis [27], we carried out hierarchical and k-means clustering. No pattern emerged from this analysis. Thus, our first attempted analysis did not yield any data in support of pre-clinical studies [32] and also demonstrated that muscle cancer cachexia appears distinct from the inflammation-driven skeletal muscle remodeling observed in the intensive care unit [27].

We then identified genes that varied with percentage weight loss using the quantitative SAM methodology [20]. In this multiple comparison corrected correlation analysis, the WS group contained both cancer patients and three non-cancer controls in order to identify *bona fide* cachexia associating genes. SAM identified 74 genes with a FDR between 0 and 10% (most <5% FDR) that covaried positively with weight loss, and nine genes with a FDR between 0 and 10% (most <5% FDR) that covaried negatively with weight loss (Additional data file 2). Correlation coefficients (R) for these 83 genes were generated using Pearson's product moment correlation. Positive coefficients ranged from 0.82 to 0.57 ($P < 0.01$), and for negatively correlating genes, R ranged from -0.74 to -0.65 ($P < 0.01$). Each relationship was visually inspected by plotting the data.

Most of the genes correlating with weight loss had not been associated previously with cachexia in humans or animal models. Notably, FOXO transcription factors and the E3 ligases MURF1 and MAFbx were absent from this list. Simple cluster analysis revealed visual distinction of patients with <5% reported weight loss from those with >5% reported weight loss (Figure 1). This Affymetrix-derived WL gene signature was technically validated by qRT-PCR of the 9 genes (*APCDD1*, *CaMKIIβ*, *EIF3I*, *HGS*, *NUDC*, *POLRMT*, *SGK*, *TIE1* and *TSC2*). Eight validated the microarray data, with only *SGK* expression being inconsistent with the Affymetrix analysis (Table 3 and Figure 2; Supplemental figure 1 in Additional data file 3).

Candidate gene approach: analysis of FOXO transcription factors and components of the ubiquitin proteasome and autophagy pathways (centre 1)

While the microarray analysis did not yield any evidence for proteolytic pathways being upregulated, as seen in intensive care unit patients with the same gene chip technology [27], investigation of components of these pathways was nevertheless undertaken in parallel to the gene-chip study. There was no difference in the nuclear level of FOXO1 and FOXO3a protein by western blotting when patients were grouped according to weight loss. Expression of the E3 ligases MURF1 and MAFbx was examined by qRT-PCR and no relationship between mRNA expression and weight loss was found (data not shown). The autophagy-related genes *GABRAPL1* and *BNIP3* were modestly increased in WL patients compared to WS patients or controls (fold change = 1.46 versus 1.23 versus 1.07, respectively; $P = 0.047$). However, this *P*-value is unadjusted for the previous array analysis and may not be reliable. Both genes demonstrated a positive association with systemic inflammation (Table S2 in Additional data file 1 and Figure S2 in Additional data file 3).

Confirmation of genes associated with weight loss in cancer cachexia (center 2)

To validate the WL gene signature generated in *rectus abdominis* muscle from the center 1 cohort, nine genes were profiled using qRT-PCR (*APCDD1*, *CaMKIIβ*, *EIF3I*, *HGS*, *NUDC*, *SKG*, *POLRMT*, *TIE1* and *TSC2*) in two additional types of skeletal muscle obtained from cancer cachexia patients. The significant association between CaMKIIβ and weight loss observed in *rectus abdominis* muscle from center 1 (R = 0.82, $P = 0.01$; Table 1) was reproduced (Figure 3a) in both *vastus lateralis* (R = 0.45, $P = 0.06$) and diaphragm muscle (R = 0.5; $P = 0.03$) from center 2 patients. In addition, *TIE1*, which significantly correlated with weight loss in *rectus abdominis* (R = 0.67, $P = 0.01$; Table 1) demonstrated a similar (Figure 3b) relationship in *vastus lateralis* (R = 0.7, $P = 0.003$) but not in diaphragm. Given the changes observed for *CaMKIIβ* mRNA, the protein and phosphorylation level of CaMKII in all of the *rectus abdominis* muscle obtained in center 1 was evaluated. Material from a total of 59 patients was available at the time the analysis was carried out (recruitment was ongoing beyond the time the microarray was carried out). Western blotting for both CaMKII (Figure 3c) and phosphorylated CaMKII (Figure 3d) revealed a small but significant ($P = 0.04$ and 0.07, respectively) increase in WL patients compared with the expression determined in WS patients and controls.

Gene interaction and promoter analysis

In order to generate valid pathway or ontological enrichment scores, it is essential to relate the modulated gene list with the genes detectably expressed in the tissue of interest and not with the genome as a whole (or the entire gene-chip content). The nature of the 83-gene WL gene signature was explored in detail using GO. The

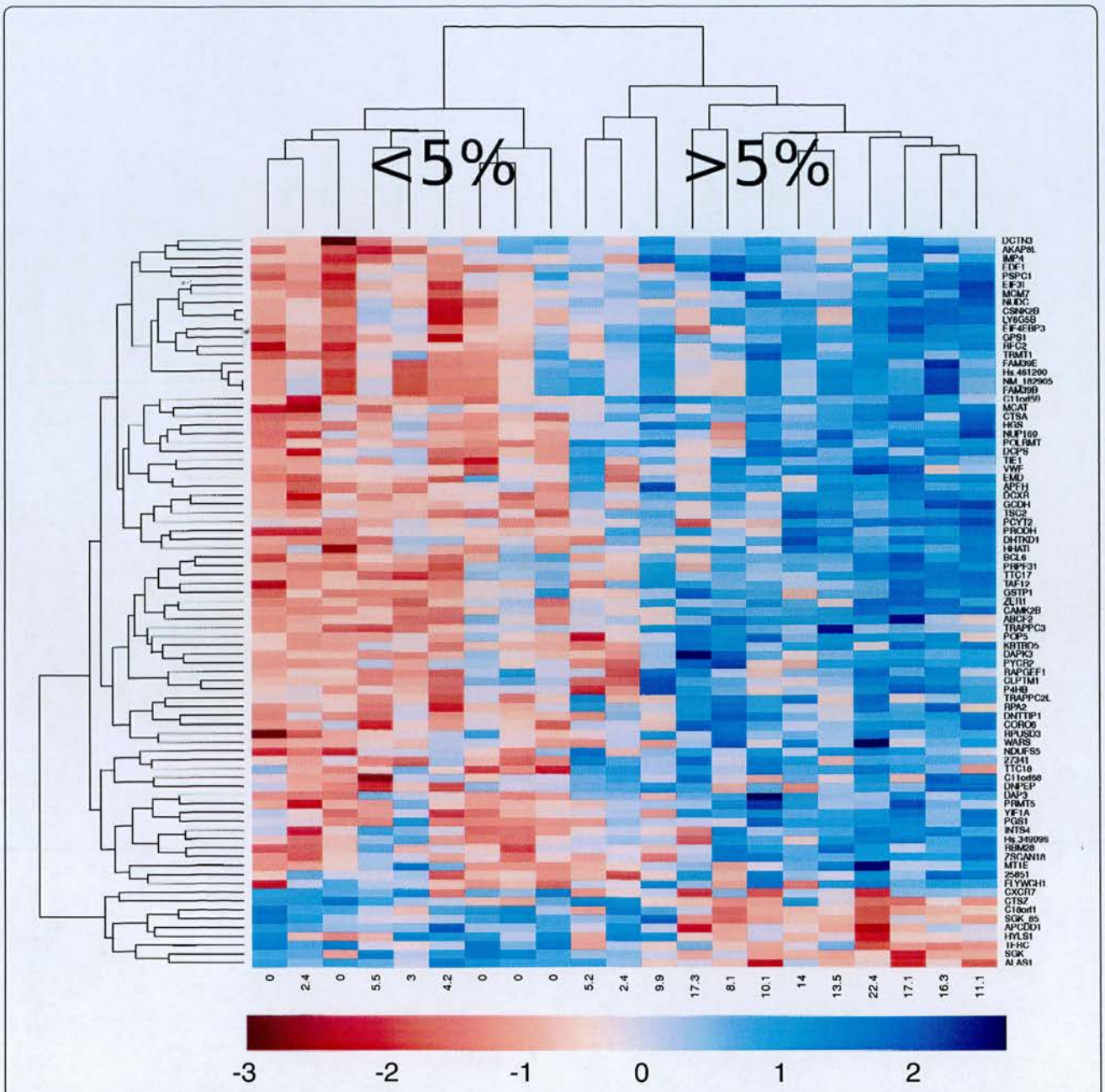

Figure 1. Cluster analysis identifies high and low weight loss groups. Using SAM and limma, 83 genes were identified as correlating with weight loss. Expression data from these genes were used to drive cluster analysis. This revealed two clusters of subjects; high weight loss (≥5%) and low weight loss (<5%).

highest ranked GO biological process activity from the DAVID webtool [33] was proline metabolism (P = 0.03). This was confirmed with the topGO [29] and GOStats [34] tools in Bioconductor. Proline metabolism has a role in collagen formation and increased collagen deposition has been noted in the muscle of cachectic cardiac failure patients [35]. Network analysis using Ingenuity [30] revealed several interactions that involve the 83 WL genes, including a calmodulin kinase gene network (Figure S3A in Additional data file 3), supporting the wet-lab data and indicating that CaMKIIβ activation appears to be a general marker of muscle wasting in human cancer cachexia. A second illustrative pathway (Figure S3B in Additional data file 3) features GLUT-4 (glucose transporter type 4) and interleukin-6, both of which are implicated in skeletal muscle metabolism [36]. This

Table 3. Genes correlating with weight loss

	Center 1 (n = 21)			Center 2 (n = 13)			
	Gene-chip			RT-qPCR			
Gene	CC *rectus abdominis*	CC *rectus abdominis*	Regression *P*-value	CC *vastus lateralis*	Regression *P*-value	CC diaphragm	Regression *P*-value
APCDD1	-0.74	-0.51	0.03	0.26	NS	-0.20	NS
CAMk2B	**0.82**	**0.50**	**0.01**	**0.45**	**0.06**	**0.50**	**0.03**
EIF3I	0.64	0.50	0.02	0.10	NS	0.20	NS
HGS	0.7	0.67	0.00	0.17	NS	0.20	NS
NUDC	0.65	0.72	0.00	0.13	NS	0.0	NS
POLRMT	0.6	0.51	0.02	0.07	NS	0.0	NS
TIE1	**0.67**	**0.53**	**0.01**	**0.70**	**0.003**	**0.0**	**NS**
TSC2	0.69	0.47	0.03	0.40	0.1	0.0	NS

Significance analysis of microarrays (SAM) identified 82 genes correlating with weight loss. qRT-PCR validated eight of nine selected targets from this list (correlation coefficient (CC)). These eight genes were also examined in the cohort from center 2 using RNA extracted from anatomically distinct regions. For each gene the correlation coefficient from the Affymetrix data set is shown followed by the correlation coefficient for qRT-PCR and a *P*-value for this latter regression. NS: not significant.

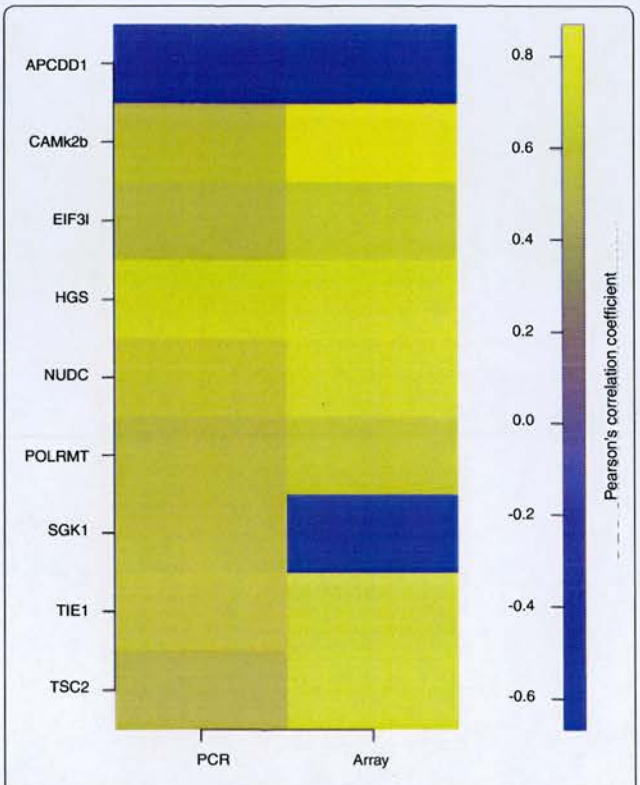

Figure 2. qRT-PCR validates array-identified genes covarying with weight loss. For each of the genes validated by qRT-PCR Pearson correlation coefficients were generated for expression and percentage weight loss for both the Affymetrix data and the qRT-PCR data. All genes except SGK1 validated the array data. P-values for the correlations ranged from 0.03 to below 0.01. Yellow indicates positive correlation; blue indicates negative correlation.

network also forms numerous connections with the glucocorticoid and androgen receptors, which may be involved in regulating skeletal muscle mass. It should be noted that despite using a back-ground gene expression file in Ingenuity [30] for genes only detected as being expressed in human skeletal muscle (approximately 21,000 probe sets, based on MAS5 present-marginal calls) the Ingenuity network analysis still included genes that may not be robustly expressed and should be used in a qualitative hypothesis generation manner.

Gene sequence analysis of the WL gene-set was carried out to provide insight into the potential coordinators of this expression signature. Interestingly, FOXO transcription binding sites tended to be, if anything, significantly under-represented in the human cachexia WL gene set, supporting the wet-lab analysis. Binding sites for SP1, ARNT.AHR (the hypoxia signaling partner) and TFAP2A (Transcription factor AP2-alpha or AP2) in particular, were over-represented in the proximal promoters of the WL-associated genes (Figure S4 in Additional data file 3). The analysis further supports the idea that this list is distinct. Interestingly, the enriched TF binding sites may function as clock genes, controlling circadian rhythm [37]. Another strategy for generating hypotheses for factors that might regulate a set of genes is to carry out comparative expression analysis [25], where two physiological studies are contrasted using global gene chip data. In this case we present data that patients with greater weight loss do not appear to have a common overlap with muscle damage, muscle degeneration in sepsis or muscle remodeling in exercise training (Figure 4).

Discussion

Cancer cachexia is thought to arise due to an imbalance of the anabolic and catabolic pathways partly driven by pro-inflammatory cytokines with consequent loss of muscle mass (along with an accompanying loss of adipose tissue). In the present study, the expression of 74 genes correlated positively with weight loss in cancer cachexia

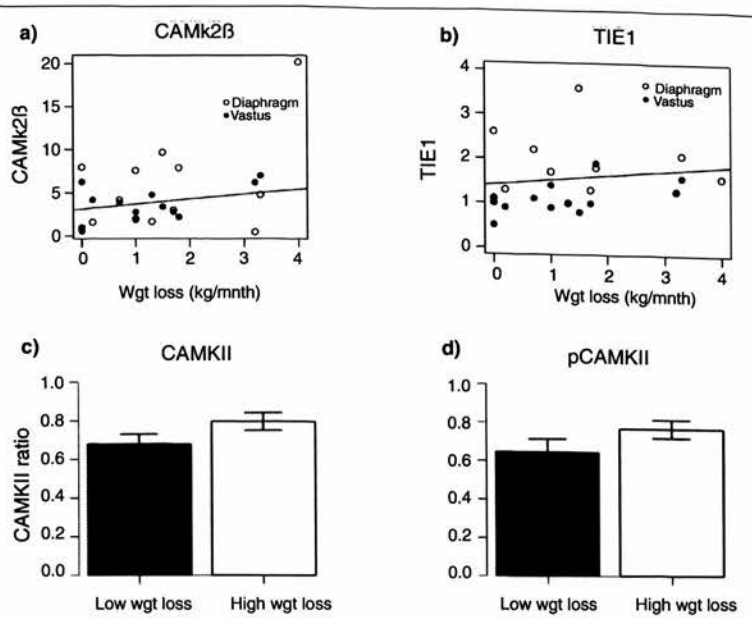

Figure 3. CAMkIIβ and TIE1 correlate with weight loss in cancer cachexia. In order to validate the findings from the *rectus abdominis*, qRT-PCR was used to examine mRNA expression of **(a)** CAMkIIβ and **(b)** TIE1 in diaphragm (open circles) and *vastus lateralis* (closed circles) in a separate clinical cohort. Correlation plots for mRNA level against rate of weight loss are shown. Correlation coefficients were significant with $P < 0.05$. CAMkII protein and phospho-protein levels are increased in subjects with weight loss. **(c)** Protein levels of CAMkII and **(d)** phosphoCAMkII were assessed in the *rectus abdominis* muscle from center 1 subjects by western blot. Intensity levels were normalized against alpha-skeletal actin and the mean ratio of CAMkII/actin or phosphoCAMkII (pCAMkII)/actin are shown for subjects with less than (black) or more than (white) 5% weight loss. *P-value <0.05, one-sided Mann Whitney test; n = 59. Error bars represent SEM.

subjects and that of 9 correlated negatively with it. Validation of these genes by qRT-PCR provided excellent technical confirmation of the microarray results. Biological validation of TIE1 and CaMKIIβ expression in an independent clinical cohort across distinct muscle groups, along with supportive network analysis, provides weight to the claim that these are useful markers of cancer cachexia in humans. Contrary to evidence from animal models [7,8,11], there were no significant differences in expression of the E3 ligases MURF1 and MAFbx, while FOXO protein activity was unchanged in WL compared to WS patients. These observations, combined with the array and promoter analysis, make it seem unlikely that FOXO transcription factors regulate the molecular signature of cachexia in human skeletal muscle, challenging the relevance of the pre-clinical literature in this field.

Novel human cancer cachexia markers

The significant correlation of *CaMKIIβ* mRNA expression with weight loss along with the small but significant change in protein levels in *rectus abdominis* suggests that CaMKIIβ could be directly involved in human cancer cachexia. *CaMKIIβ* mRNA also increased with weight loss in *vastus lateralis* and diaphragm. The serine/threonine kinase CaMKII holoenzyme is activated by Ca^{2+}/calmodulin, leading to autophosphorylation and maintenance of CaMKII activity even after the Ca^{2+} signal has diminished [38]. CaMKIIβ is expressed in skeletal muscle, and levels of the protein as well as its phosphorylation status and activity increase after exercise training [39]. The relationship between CaMKIIβ expression and cachexia observed in the present study implies that the cancer cachexia profile is not simply 'physical inactivity'. In addition, it has recently been demonstrated that Ca(2+)-CaM-eEF2K signaling may be responsible for acute exercise-induced inhibition of muscle protein synthesis [40] and it is thus conceivable that chronic inappropriate activation of this 'endurance training'-related signaling molecule [24] subdues normal maintenance of skeletal muscle mass. Additional factors that could modulate CaMKII activity include alterations in lipid metabolism [41].

The significant positive correlation for *TIE1* mRNA expression with weight loss in both the *rectus abdominis* and *vastus lateralis* muscle groups supports the idea that some chronic training-related genes are up-regulated in cachexia. In animal models TIE1 is required for normal vascular network development [42] while increased *TIE1* mRNA levels in human skeletal muscle in response to physiological adaptation to exercise training has been demonstrated [43]. Whilst the ligands and signaling

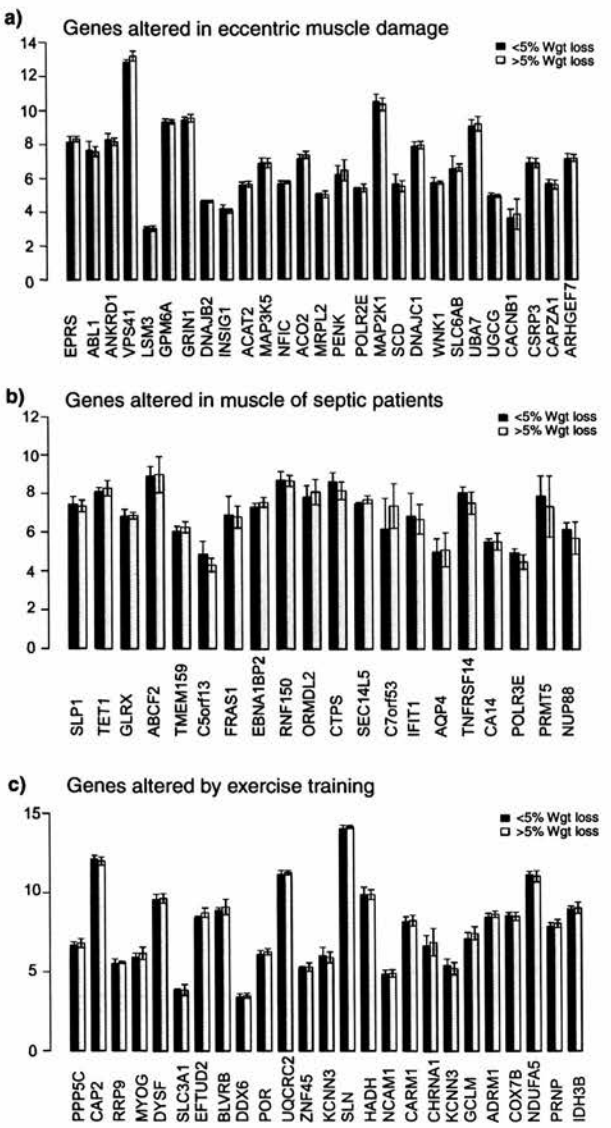

Figure 4. Gene expression signatures demonstrate lack of relationship between weight loss and muscle damage, muscle sepsis and exercise training status. The top 20 most regulated genes by **(a)** eccentric muscle damage, **(b)** muscle obtained from intensive care unit patients and **(c)** in response to exercise training were obtained from three published articles (see Methods). The mean values for these selected genes were then plotted for patients in the present study that had either less than or more than 5% weight loss. As can be observed, no single gene, for each of these 'comparative' conditions, was differentially expressed; thus, the gene expression profile of cancer cachexia does not resemble muscle damage, sepsis-induced degeneration or exercise training status. Error bars represent SEM.

pathways of TIE1 are poorly understood, this receptor can interact with phosphoinositide 3-kinase and lead to phosphorylation and activation of Akt, protecting cells from apoptosis [44]. In functional terms, the up-regulation of TIE1 may therefore represent a protective mechanism to oppose apoptosis of some components of skeletal muscle tissue, for example, the vascular endothelium. TIE1 has also recently been linked to an *in vitro* endothelial inflammatory response [45] while an inflammatory gene signature has been shown to develop throughout surgical procedures in muscle [46]; thus, it could be argued that some component of our gene signature may be related to surgery. However, all biopsies were taken at the earliest point in surgery after the initial incision.

Furthermore, the correlation of TIE1 expression with weight loss and the lack of any further appreciable inflammatory signature would argue against this possibility. In addition, there was no evidence that the muscle profile was that of damage or that observed with systemic inflammation associated with multiple organ failure (Figure 4). It is also notable that (other than *TIE1*, *CaMKII*, *CTSA* and *PRODH*) the WL gene signature does not share similarities with the approximately 500-gene endurance exercise training gene signature [24], suggesting that the reason for elevated TIE1 and CaMKIIβ remains to be determined. It may be inappropriate partial muscle activity signaling but clearly is not simply increased muscle usage (however unlikely that might have seemed in such patients). However, the increased *CaMKIIβ* mRNA levels associated with weight loss across a range of muscle tissues imply that these muscle groups develop dysregulation of calcium sensing or are burdened by greater loading in the face of failing muscle function connected with, for example, loss of contractile machinery or impaired energy metabolism [47].

Finally, recent work has clarified two potential calcium-independent activation pathways for CaMKII. Generation of reactive oxygen intermediates can increase or prolong CaMKII activity, perhaps through inhibition of protein phosphotases that normally limit CaMKII activation [48]. CaMKII has also been implicated in muscle adaptation through phosphorylation of HDAC5 leading to MyoD/MEF2-driven differentiation of muscle cells [49]. It is plausible, therefore, that CaMKII activation is a compensatory strategy in the face of failing protein synthesis. Alternatively, the CaMKIIβ response may indicate failure of calcium homeostasis, a factor that would also activate proteolytic activities such as calpains and caspases [50,51]. It is thus possible that CaMKIIβ activation occurs at an early stage of cachexia in humans, providing an early 'read-out' on altered calcium handling.

Human versus animal-model cancer cachexia markers and study limitations

Given the robust increase in expression of the E3 ligases reported previously in various animal models of cachexia [7,8,32], it is surprising that neither microarray nor

qRT-PCR detected any regulation of MuRF1 and MAFbx. Furthermore, the 83-gene WL gene signature bore no resemblance to the Atrogene gene expression signature [27,32] generated using gene-chips. This is not due to gene-chip technology being unable to establish parallels between animal models and humans, as it has previously been demonstrated that gene expression in skeletal muscle of intensive care unit patients resembles, in part, that found in these animal models [27,32]. Indeed, results of E3 ligase expression analysis from other human models of cachexia have been contradictory. Studies including patients following bed rest, amputation for vascular disease, limb immobilization, chronic obstructive pulmonary disease, amyotrophic lateral sclerosis and ageing have demonstrated both increased and decreased expression of MuRF1 and MAFBx [52-56]. This would suggest that the ubiquitin E3 ligases do not play the same role in human cancer cachexia as that previously demonstrated in animal and cell studies. In lung cancer patients with mean weight loss of 2.9%, there was no evidence of UPP activation [57] while other human studies in patients with gastric cancer and mean weight losses of 5.2% and 5.6% have shown increases in components of the UPP [58,59]. In the present study we could not find any support for this finding, despite similar degrees of cachexia. However, cancer cachexia encompasses a spectrum progressing from early weight loss through to severe muscle wasting. The prominence of the individual proteolytic pathways at different time points along this spectrum is yet to be determined and one must keep in mind that during severe tissue wasting, both breakdown (and of course synthesis) may well be reduced with the net balance between the two widened.

A role for autophagy in human cancer cachexia has not been investigated extensively. Increased cathepsin D and acid phosphatase activity has been demonstrated in patients with varying tumor types and degrees of weight loss, suggesting that increased lysosomal activity may contribute to the development of cachexia [60]. More recently, lung cancer patients undergoing resection were shown to have increased levels of cathepsin B mRNA in skeletal muscle compared with controls [57]. The analyses examined GABARAPL1 and BNIP3. GABARAPL1 is an Atg8 homologue important in the formation of the autophagosome [61] and BNIP3 has been found to play a predominant role in induction of autophagy in rodent skeletal muscle [11]. Autophagy can be induced by starvation of amino acids, which may explain the modest increase in BNIP3 and GABARAPL1 in patients with SI where the acute phase response is activated (mobilizing amino acid from muscle to liver for consumption) and where food intake may be reduced due to anorexia or dysphagia. However, no relationship was found between these genes and patient weight loss.

A limitation of the current study is that we focus on changes in total body mass and this does not tell us about the relative contributions from lean body mass and adipose tissue. Our muscle gene expression clustering results indicate, however, that there is a skeletal muscle molecular signature that reflects changes in whole body mass and it is hard to conceive that this is not somehow reflecting the changes in the muscle tissue. A further consideration is adequate control for confounding parameters, such as inflammation, damage and physical activity. While these are difficult to directly control, we produced an analysis to suggest that such processes were unrelated to our new human muscle cancer cachexia signature (Figure 4).

Conclusions

Human cancer cachexia is a chronic process and weight loss is not as rapid and generally not as severe as the acute muscle wasting observed in animal models. Thus, the physiological regulators are most likely very distinct in each scenario. We found increased expression of two 'endurance exercise'-activated genes, *CaMKIIβ* and *TIE1*, across different muscle groups in human cancer cachexia. Whether these could contribute to a reduction in protein synthesis remains to be ascertained.

Additional data file 1. Primers used in the study, genes associated with systemic inflammation and data on autophagy pathway genes.

Additional data file 2. Genes associated with weight loss or systemic inflammation in cancer cachexia.

Additional data file 3. Figures and figure legends for supplementary figures referred to in the text.

Abbreviations

BMI: body mass index; CaMK: calcium/calmodulin-dependent protein kinase; DTT: dithioreitol; FDR: false discovery rate; GO: Gene Ontology; MAS 5.0: Microarray Suite; PMSF: phenylmethanesulfonyl fluoride; qRT-PCR: quantitative reverse transcriptase PCR; SI: systemic inflammation; SAM: significance analysis of microarrays; TBS: tris-buffered saline; TBST: TBS with Tween 20; UPP: ubiquitin proteosome pathway; WL: weight losing; WS: weight stable.

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Authors' contributions

The genomics analysis strategy and statistical analysis was developed and carried out by JAT and IJG. Wet-lab genomic analysis was carried out by IJG,

NAS, TM, OR and JAT. Western analysis was carried out by NAS, DCG and JAR. The manuscript was drafted by JAT and IJG. The manuscript was edited by IJG, NAS, JAT, TM, OR, JAR, DCG and KCHF. The clinical biobank materials were established by RJES, KCHF, NAS, LL, OR and BT. All authors have given final approval to the article.

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Competing interests
This project was assisted in part by an Affymetrix Translational Medicine award (JT) that reduced the cost of the gene-chip analysis. Affymetrix were not involved in any aspect of the data analysis or interpretation and did not influence the manuscript in any way. The authors declare that they have no competing interests.

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Cytokine gene polymorphisms and susceptibility to cachexia

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Purpose of review

Cachexia is a progressive deterioration of body habitus associated with chronic diseases. The finding that only a proportion of patients with chronic disease develop cachexia has prompted studies looking for genetic polymorphisms that may underlie differential susceptibility. The aim of this review is to explore how inflammation and gene polymorphisms influence susceptibility to cachexia.

Recent findings

There has been evidence that certain cytokine gene polymorphisms are associated with cachexia. However, only the *IL10* −1082 G allele, which is associated with an increased risk of developing cachexia has been replicated in more than one study. Variation in genes outwith inflammation pathways (e.g. genes involved in protein metabolism) is also likely to contribute the susceptibility of developing cachexia. The insertion/deletion angiotensin converting enzyme (*ACE*) gene polymorphism has recently been linked with lower lean body mass in cancer patients with cachexia.

Summary

Although there is an increasing body of evidence of genetic susceptibility to cachexia, most studies so far have only focussed on a small number of polymorphisms and have small sample sizes. Large-scale candidate gene studies or genome-wide association studies are required to further elucidate the link between genotype and cachexia.

Keywords

cachexia, genetics, inflammation, polymorphisms

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Introduction

Cachexia is a complex syndrome that combines weight loss, loss of muscle and adipose tissue, anorexia, and weakness [1]. The origin of the term 'cachexia' is from the Greek words kakos (bad) and hexis (condition or appearance) and throughout medical history has been associated with the gravely ill patient. Cachexia significantly impairs quality of life (QoL) and response to treatment and is associated with increasing morbidity and mortality. Cachexia typically manifests in chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), and chronic kidney disease (CKD) [1].

Inflammation in chronic diseases

An organism usually responds to the presence of infection, tumour, immunological disorders, tissue injury, trauma, or surgery by developing an inflammatory response that may help limit tissue injury. However, in certain circumstances when the inflammatory response is prolonged or severe, it can lead to detrimental effects.

The idea that cancer and inflammation are linked has been around since the hypothesis of Virchow in the 19th Century [2]. Apart from a role in cancer development and progression, cancer-associated inflammation may also contribute to the development of cachexia [3••].

The inflammatory response in the lungs is central to the structural changes leading to COPD. Studies show that reduced lung function is associated with elevated systemic inflammatory factors that increase during exacerbations [4], and these factors may contribute to the comorbidities observed in patients with COPD such as cachexia.

Heart failure is associated with elevated circulating levels of inflammatory mediators. In the Framingham Study, elderly individuals with no history of myocardial infarction or congestive heart failure had a significant increase in congestive heart failure risk per tertile increment in cytokine concentration [5]. The presence of a more pronounced inflammatory response has also been observed in patients with cardiac cachexia [6].

Inflammation and cachexia are prevalent in patients with CKD and worsen as the CKD progresses toward end-stage renal disease (ESRD) [7]. The deterioration of renal function has been associated with increased serum cytokine levels and their soluble receptors in patients with varying degrees of renal failure [8,9••].

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The role of inflammation in cachexia

Cachexia is brought about by a synergistic combination of a dramatic decrease in appetite and an increase in metabolism of fat and lean body mass. Although the specific molecular mechanisms leading to the development of cachexia are not fully understood, systemic inflammation appears to play a central role [10] (Fig. 1). Inflammation results in a number of metabolic changes that are often characterized by negative energy balance, increased thermogenesis and anorexia. These responses are mediated by cytokines [11,12•].

Anorexia

Cytokines produced during inflammation can affect the brain by several pathways: they can act on peripheral tissues producing hormones such as leptin which influence the activity of the brain; they can act on peripheral neurons that project to the brain through the vagus nerve; and they can also directly enter the brain and act locally [13].

Many cytokines are known to have an effect on appetite, including interleukin (IL)-1α, IL-1β and IL-6 as well as tumour necrosis factor-α (TNF-α) [14]. IL-1, in particular, has been clearly associated with the induction of anorexia [15], by blocking neuropeptide Y (NPY)-induced feeding. The levels of this molecule (a feeding-stimulating peptide) are reduced in anorectic tumour-bearing rats and a correlation between food intake and brain IL-1 has been found in anorectic rats with cancer [16].

Increased energy expenditure

Resting energy expenditure (REE) is significantly higher in those with an elevated acute phase response (APR) [17]. The APR is a series of changes in liver protein synthesis, which shifts from production of albumin to acute phase proteins (APP), such as C-reactive protein (CRP), fibrinogen, serum amyloid A, and α-1 antitrypsin, in response to an insult such as inflammation.

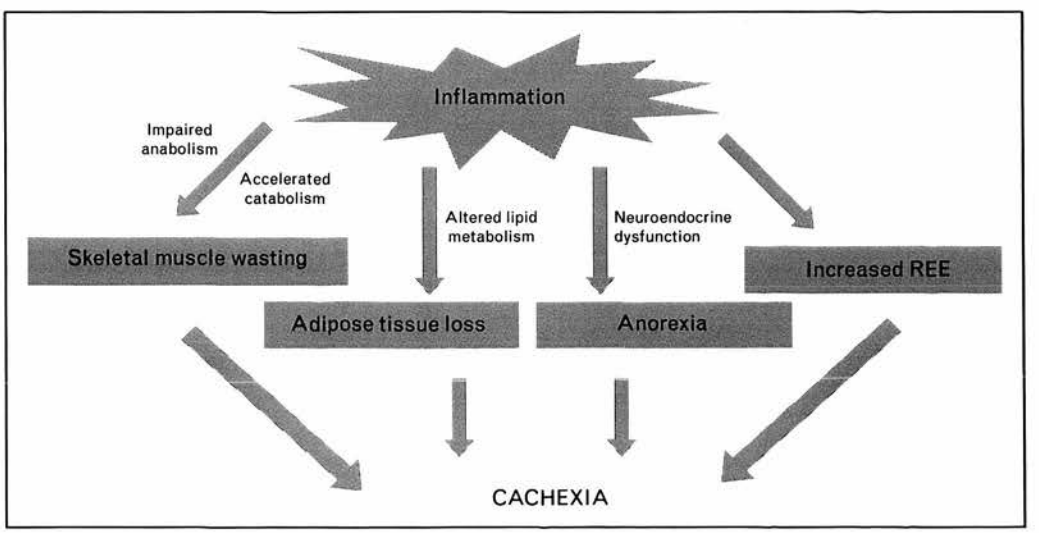
There is a known association between the development of an APR and the rate of loss of body mass in lung and gastrointestinal cancers [18], and in patients with non-small cell lung cancer (NSCLC), serum levels of IL-6 were found to correlate with concentrations of circulating CRP [19].

Altered lipid metabolism

The multifunctional cytokine, TNF-α exerts a series of biological actions in different cells, tissues, organs and species and has been demonstrated to regulate and interfere with energy metabolism, especially lipid homeostasis. A large body of research suggest that the effects of TNF-α on lipid metabolism mainly include five aspects: suppression of free fatty acid (FFA) uptake and promotion of lipogenesis; induction of lipolysis; inhibition of lipid-metabolism-related enzyme activity; regulation of cholesterol metabolism; and regulation of other adipocyte-derived adipokines [20•].

Although TNF-α is the first cytokine reported to have catabolic effects on fat cells, subsequent studies have shown that multiple cytokines acting through multiple receptors have similar activities. TNF-β, interferon-α, interferon-γ, IL-1 and IL-6 decrease lipoprotein lipase activity and increase lipolysis in cultured fat cells [21]. TNF-β and both interferons also decrease de-novo fatty

Figure 1 Role of inflammation in cachexia



Inflammation is thought to influence pathways leading to skeletal muscle wasting, adipose tissue loss, anorexia, and increased resting energy expenditure (REE).

acid synthesis in cultured fat cells [21]. The catabolic effects of cytokines on adipose tissue triglyceride metabolism have been proposed as a contributor to the development of cachexia [3••].

Impaired protein synthesis

Inflammatory-mediated signalling may limit muscle protein synthesis by several mechanisms. Inflammatory cytokines such as TNF-α and IL-6 are likely to play an important role. TNF-α can activate the transcription factor nuclear factor-kappa B (NF-κB), which inhibits the synthesis of the muscle specific transcription factor MyoD, thereby inhibiting differentiation [22]. In addition, a recent study found that skeletal muscle protein synthesis was dramatically reduced by relatively low-dose IL-6 infusion in humans [23].

Preliminary reports have described marked elevations in myostatin (a negative regulator of muscle mass that inhibits myogenic proliferation and differentiation) in cachetic cancer patients. Animal models suggest that the inflammatory cytokine TNF-α is at least partially responsible for this increase [24]. TNF-α is also known to influence the anabolic mTOR signalling pathway, which is a major mediator of anabolic responses in skeletal muscle [25].

IL-15 is a cytokine that is highly expressed in skeletal muscle [26]. IL-15 is anabolic in nature and acts by activation of protein synthesis and concomitant inhibition of protein degradation in cultured skeletal muscle cells [27]. IL-15 administration in an in-vivo rodent model of cancer cachexia has been shown to inhibit skeletal muscle wasting [28]. Muscle and serum IL-15 levels are thought to be reduced in cachexia [29].

Increased protein degradation

Inflammatory stimulation activates pathways associated with muscle protein breakdown [30•]. In cancer cachexia, the ubiquitin proteasome pathway is greatly influenced [31]. Many of these effects are thought to occur through activation of NF-κB by upstream factors such as TNF-α [32]. NF-κB stimulates transcription of the ubiquitin E3 ligase muscle ring finger (MuRF)-1, which is known to positively regulate activity of the ubiquitin proteasome pathway [33].

Genetic susceptibility to cachexia

The finding that only a proportion of patients with chronic disease develop cachexia has prompted studies looking for genetic polymorphisms that may underlie differential susceptibility [34]. The most frequent targets for these studies have been genes encoding pro-inflammatory cytokines, such as TNF-α and some of the interleukins. This is based on the hypothesis that continued systemic inflammation plays a central role to the pathogenesis of cachexia (see above).

One of the earliest genetic susceptibility studies by Broekhuizen *et al.* [35] found that a −511 polymorphism in the *IL1B* gene (rs16944) correlated strongly with cachexia in patients with COPD. The C/C genotype at this position in the gene was associated with frequent cachexia, whereas the T/T genotype was not associated with cachexia (Table 1).

Table 1 Summary of cytokine gene polymorphisms associated with cachexia

Gene	Polymorphism	Minor allele frequency[a] European	Minor allele frequency[a] Chinese	Clinical studies
IL-1B	511T > C [rs16944]	64%	54%	C/C genotype associated with frequent cachexia in patients with COPD ($n = 99$) [35]
	3954C > T [rs1143634]	21%	2%	T allele more prevalent in gastric cancer patients with cachexia; C/T genotype associated with OR 2.5 of developing cachexia ($n = 214$) [36]
				Patients with metastatic gastric and GOJ cancer with C/T and T/T genotype showed greater improvements in weight over time ($n = 44$) [37]
IL-10	1082G > A [rs1800896]	53%	5%	G allele associated with increased weight loss in patients with gastro-oesophageal cancer; G/G genotype associated with OR 2.3 of developing cachexia ($n = 203$) [38]
				G allele more prevalent in gastric cancer patients with cachexia; A/G genotype associated with OR 2.0 of developing cachexia ($n = 223$) [39]
	819C > T [rs1800871]	17%	74%	C/C genotype associated with OR 3.4 of developing cachexia in gastric cancer patients ($n = 223$) [39]
IL-6	634G > C [rs1800796]	4%	77%	G allele more prevalent in pancreatic cancer patients with cachexia ($n = 126$) [40]
IL-8	781C > T [rs2227306]	4%	37%	T allele more prevalent in gastric cancer patients with cachexia; T/T genotype associated with OR 3.0 of developing cachexia ($n = 125$) [41]

COPD, chronic obstructive pulmonary disease; GOJ, gastro-oesophageal junction; OR, odds ratio.
[a]Minor allele frequency data obtained from International HapMap project database (http://hapmap.ncbi.nlm.nih.gov).

Although this finding has not been confirmed in subsequent studies, Zhang *et al.* [36] have found that a separate polymorphism within the *IL1B* gene is associated with cachexia. Gastric cancer patients in China with cachexia showed a significantly higher prevalence of the (rs1143634) *IL1B* +3954 T allele than those without, and the *IL1B* +3954 C/T genotype was associated with an 2.5 times increased risk of developing cachexia (Table 1). Unfortunately, the reverse association was found in another study involving patients with gastric and gastro-oesophageal junction cancer [37]. Patients with the *IL1B* +3954 C/T and T/T genotypes showed greater improvements in their weight and in survival over time than did patients with the C/C genotype (Table 1).

We have recently investigated the relationship between five cytokine polymorphisms and markers of nutritional status among patients with gastro-oesophageal cancer [38]. Possession of the (rs1800896) *IL10* −1082 G allele was found to be associated with increased weight loss and the G/G genotype was associated with a 2.3 times increased risk of developing cachexia (Table 1). In a separate study involving patients with gastric cancer in China, Sun *et al.* [39] confirmed that the G allele was more prevalent in patients with cachexia. The study also found that individuals with the (rs1800871) *IL10* −819 C/C genotype were at increased risk of developing cachexia (Table 1).

A further two cytokine polymorphisms have been associated with cachexia. The (rs1800796) *IL6* −634 G allele has been shown to be significant more prevalent in Chinese pancreatic cancer patients with cachexia compared with those without cachexia [40] (Table 1). In patients with gastric cancer in China, the frequency of (rs2227306) *IL8* +781 T allele was noted to be significantly increased in patients with cachexia and the +781 T/T genotype was observed to be associated with a significantly increased risk of cachexia [41] (Table 1).

It is perhaps worth noting that the minor allele frequencies (MAFs) of polymorphisms in the studies mentioned above vary across different populations (Table 1). MAF distributions for polymorphisms assessed in the Chinese-based studies were significantly different in most cases compared with European populations. These variations in gene allele frequencies can contribute to differences in the risk of developing complex conditions like cachexia among different populations [42•].

Although much attention has been placed on cytokine polymorphisms and their association with cachexia, it is important to note that mechanisms surrounding the pathogenesis of cachexia is complex and is likely to involve gene products with act both upstream and downstream of cytokines. Pathways not affected by cytokines may also play a role in the development of cachexia. It is therefore highly likely that genetic variations in non-cytokine genes may also contribute to the susceptibility of developing cachexia. For example, a study by Vigano *et al.* [43•] has shown that the angiotensin-converting enzyme gene (*ACE*) insertion/deletion polymorphism (rs4646994) is associated with lower total fat mass and lean body mass in patients with gastro-intestinal cancer and NSCLC.

A recent gene microarray study by Stephens *et al.* [44••] on muscle samples of cachectic gastro-oesophageal cancer patients revealed 83 new candidate genes associated with the development of cachexia. Another study on regulatory gene pathways that accompany loss of adipose tissue in cancer cachexia revealed 71 downregulated genes and five upregulated genes [45••]. Variants in genes discovered in both these studies could well contribute to the differential susceptibility to cachexia.

The search for altered genes associated with cachexia is still in its infancy and, even though some associations have surfaced, cause and effect remains to be established. The studies are also plagued by a variety of problems, in particular phenotype misclassification (which is highly relevant to cachexia where there is no validated definition of the syndrome). It is worth noting that some studies have small sample sizes with inadequate statistical power. In addition, epistatic interaction, and undetected genomic heterogeneity in the underlying population also exist [34]. Of note, in a recent review of allelic association with common disease phenotypes, only 6 of 166 associations subjected to multiple evaluations were confirmed consistently [46]. Possible causes of false-positive association studies include population stratification, variable linkage disequilibrium and genotype misclassification. In addition, in many of these studies, the possible effects of single gene variants were assessed in situations when combined impacts of multiple factors could be expected [47].

It should also be emphasized that although certain variant combinations can predispose to a condition, others may be protective against the same condition. For example, polymorphisms result in an increase in systemic inflammation, a decrease in lean or fat mass and decreased survival in cancer are likely to be more cachexia-prone variants. On the contrary, polymorphisms that result in a decrease in systemic inflammation, an increase in body mass and improved survival in cancer are likely to be less cachexia-prone variants.

Conclusion

Although there is an increasing body of evidence of genetic susceptibility to cachexia, most studies so far

have only focussed on a small number of polymorphisms and have small sample sizes. It can be assumed that analysis of combinations of gene variants encoding interacting factors within a biological chain or cascade, rather than isolated investigation of its single components, may have more chances to reveal real causative connections between gene polymorphisms and phenotypes; therefore, large-scale candidate gene studies or genome-wide association studies are required to further elucidate the link between genotype and cachexia.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 297).

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REVIEW ARTICLE

Identification of possible genetic polymorphisms involved in cancer cachexia: a systematic review

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Abstract

Cancer cachexia is a polygenic and complex syndrome. Genetic variations in regulation of the inflammatory response, muscle and fat metabolic pathways, and pathways in appetite regulation are likely to contribute to the susceptibility or resistance to developing cancer cachexia. A systematic search of Medline and EmBase databases, covering 1986–2008 was performed for potential candidate genes/genetic polymorphisms relating to cancer cachexia. Related genes were then identified using pathway functional analysis software. All candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms associated with cachexia using the OMIM and GeneRIF databases. Genes with variants which had functional or clinical associations with cachexia and replicated in at least one study were entered into pathway analysis software to reveal possible network associations between genes. A total of 184 polymorphisms with functional or clinical relevance to cancer cachexia were identified in 92 candidate genes. Of these, 42 polymorphisms (in 33 genes) were replicated in more than one study with 13 polymorphisms found to influence two or more hallmarks of cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival). Thirty-three genes were found to be significantly interconnected in two major networks with four genes (*ADIPOQ*, *IL6*, *NFKB1* and *TLR4*) interlinking both networks. Selection of candidate genes and polymorphisms is a key element of multigene study design. The present study provides an initial framework to select genes/polymorphisms for further study in cancer cachexia, and to develop their potential as susceptibility biomarkers of developing cachexia.

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Introduction

Cancer cachexia is a multi-factorial syndrome characterized by chronic wasting involving loss of both adipose tissue and lean body mass (LBM). Depending on the tumour type, weight loss occurs in 30%–80% of the cancer patients and is severe (with loss of >10% of the initial body weight) in 15% (Dewys *et al.* 1980). Although certain tumour types are more commonly associated with cachexia, even with the same tumour type there are variations in the extent to which patients exhibit cachexia. Such variation maybe, in part, due to the patient's genotype rather than the tumour phenotype and/or tumour interaction. It is therefore likely there may be cachexia prone genotypes as well as cachexia resistant genotypes.

The cachexia syndrome is thought to result from a complex interplay of mechanisms involving the initiation of a host inflammatory response mediated by tumour-derived proinflammatory cytokines; the reprioritization of protein metabolism with induction of the acute phase response and mobilization of fat reserves; and activation of neuroendocrine pathways which may lead to hypermetabolism and increased catabolism (Tan *et al.* 2008). The wealth of known genetic variation in genes regulating the above mechanisms suggests their exploitable potential as biomarkers of inter-individual predictability of developing cachexia.

Single nucleotide polymorphisms (SNPs) are the most common type of stable genetic variations in the population

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(Brookes 1999). SNPs occur in approximately one in every thousand bases. There are several ways that SNPs can lead to an aberrant gene product. Promoter polymorphisms that alter DNA binding of transcription factors have the potential to decrease or increase gene expression; sequence variation in the 5′ untranslated region (UTR) could disrupt mRNA translation, and mutations in the 3′ UTR could affect mRNA cleavage, stability and export; finally, nonsynonymous SNPs in exons could alter protein function or activity. It has been estimated that 10% of all SNPs in the genome are functional, thereby having the potential of altering some biological process (Wjst 2004).

Over the past 10 years, considerable information has emerged on mechanisms of cancer cachexia of which inflammation is postulated to play a significant role. These mechanisms can be broadly grouped into five domains of interest: systemic inflammation, central energy balance, control of muscle metabolism/function, control of adipose tissue metabolism/function, and regulation of appetite.

This systematic literature review aims to explore genetic polymorphisms with known functional or clinical significance in potential candidate genes involved in the development of cancer cachexia within the above-mentioned domains. It also aims to identify the polymorphisms with the most likely potential as susceptibility markers for cancer cachexia.

Methods

The scientific literature published between 1986 and 2008 was searched in Medline and EmBase databases. In order to maximize the potential of identifying potential candidate genes, we utilized search terms that took into account the effects of cachexia. For example, 'survival' as a surrogate for accelerated death which may be due to cachexia. The term 'body composition' was used to identify potential genes of interest that may predispose individuals to higher/lower body mass which may influence the propensity to the development of cancer cachexia. 'Inflammation' was used as a main search term due to its postulated central role in the development of cancer cachexia. Overall, the following search terms were used to identify potential candidate genes/polymorphisms of interest: ((genes/genetics) or polymorphism(s)) and (inflammation or cancer or cachexia or weight loss or body composition or survival). The search was limited to studies on humans, in English language.

Following the initial retrieval of possible candidate genes, the genes were entered into a pathway functional analysis software (Ingenuity Pathways Analysis (IPA), Ingenuity Systems, Redwood, USA) to further identify related genes.

All identified candidate genes were then reviewed for functional polymorphisms or clinically significant polymorphism in terms of cachexia using OMIM and GeneRIF databases. Candidate genes were grouped into categories according to genes that regulate or code for the following:

- Inflammation
    - Innate immune receptors and mediators of the immune response
    - Cytokines
    - Cytokine receptors and related binding proteins
    - Acute phase protein reactants
- Central homeostasis
    - Energy production
    - Insulin like growth factors and related proteins
    - Corticosteroid signalling proteins
- Muscle
    - Muscle function and structure
    - Muscle proteolysis
- Adipose tissue
    - Adipogenesis
    - Lipid turnover and transport
    - Adipokines and adipokine receptors
- Appetite
- Others

Summary tables of polymorphisms are also presented according to each category with 'easy to see' boxes that denote whether a polymorphism has any effect on inflammation, weight/body composition (i.e. lean mass/fat mass) and cancer survival. The summary tables also denote if a functional or clinical association with a polymorphism was replicated in more than one study. In addition, polymorphism reference numbers (rs numbers) were also recorded if known, as well as the minor allele frequency of the polymorphism based on a population with European ancestry derived from the HapMap or dbSNP databases.

Pathway analysis

It is likely that single genes are not adequate by themselves to affect disease risk; however, multiple genes within a single pathway may affect function enough to increase risk. To provide a more comprehensive assessment in terms of pathway involvement in cancer cachexia, we performed pathway-based analyses using the ingenuity pathway analysis (IPA) software. Based on the systematic literature review, the genes with variants that had functional or clinical associations replicated in at least one study were entered into the IPA analysis tool. These genes were termed focus genes. The IPA software was used to measure associations of these genes with other molecules, their network interactions, and biologic functions stored in its knowledge base. Our focus

genes served as seeds for the IPA algorithm, which recognizes functional networks by identifying interconnected molecules, including molecules not among the focus genes from the IPA knowledge base. The software illustrates networks graphically and calculates a score for each network, which represents the approximate 'fit' between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the –log (Fisher's exact test result).

Results

A total of 184 polymorphisms with functional and/or clinical significance in terms of cachexia were identified in 92 genes.

Inflammation

Inflammation has long been associated with cancer cachexia. Systemic inflammation could result from tumour cell or host-cell-mediated production (Stewart *et al.* 2006). In experimental models, pro-inflammatory cytokines such as interleukin 1-beta (IL-1β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNFα), and interferon gamma (IFNγ) may lead to an acute phase response and trigger tissue catabolism (Argiles *et al.* 2003).

Innate immune receptors and mediators of the immune response

Table 1 of electronic supplementary material at http://www.ias.ernet/jgenet/ explores the possible genetic determinants in the generation or suppression of the inflammatory response and how they may relate to cancer cachexia. These include variants in gene coding for the Toll-like receptor (TLR) family, and associated genes, which play an instructive role in innate immune responses as well as the subsequent induction of adaptive immune responses. TLRs are involved in triggering intra-cellular signals, culminating in the activation of nuclear factor (NF)-κB, where it participates in enhancing expression of other immuneregulatory substances (Kawai and Akira 2006). NF-κB is a transcriptional regulator that plays a central part in responses to inflammatory signalling. Polymorphisms in genes encoding for NF-κB and genes involved in the activation or inhibition of NF-κB are also shown in table 1 of the electronic supplementary material. Also of interest are variants in genes coding for cell adhesion molecules (CAMs) that are proteins located on the cell surface involved in binding with the other cells or with the extracellular matrix. CAMs are known to mediate migration of cells to sites of inflammation. Functional polymorphisms in the heat-shock proteins genes HSPA1L and HSPA1B have been noted in relation to inflammation and these are also displayed in table 1 of electronic supplementary material.

Cytokines and cytokine receptors

Cytokines are secreted proteins that play a role in the induction and effector phases of all immune and inflammatory responses. They serve diverse functions including induction of cell proliferation, mediating intercellular communication, chemotaxis and cell killing. Genetic variants of genes encoding pro-inflammatory and anti-inflammatory cytokines are presented in table 2 of electronic supplementary material.

Cytokines exert their effects via matching cell-surface receptors. Subsequent cascades of intracellular signalling then alter cell functions. This may include the upregulation and/or downregulation of several genes and their transcription factors, resulting in the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition (Ihle 1995). Table 3 of electronic supplementary material summarizes the variations in genes encoding cytokine receptors and related binding proteins.

Acute phase protein reactants

Acute-phase protein reactants (APPR) are proteins whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation (Stephens *et al.* 2008), and are also predictors of adverse outcomes in cancer patients. Some APPR also have roles in modulating the immune response such as C-reactive protein (CRP). Variants in genes coding for APPR are shown in table 4 of electronic supplementary material.

Central homeostasis

Body mass is controlled by the balance of energy intake and expenditure, like all thermodynamic systems. In certain forms of cancer, patients with cachexia have been observed to have much higher resting energy expenditure (REE) (Fredrix *et al.* 1991). Gene polymorphisms in the regulatory pathways controlling energy intake and expenditure are discussed below. The following section also explores genes involved in growth and development, and metabolic pathways common to both muscle and adipose tissues.

Energy production

Uncoupling proteins (UCPs) are transporters, present in the mitochondrial inner membrane, that mediate a regulated discharge of the proton gradient that is generated by the respiratory chain. This energy-dissipatory mechanism can serve functions such as thermogenesis, maintenance of the redox balance, or reduction in the production of reactive oxygen species (Ledesma *et al.* 2002). There are a total of five UCP homologs in humans. There are known significant polymorphisms within three UCP genes that may have a role in the development of cachexia and these are presented in table 5 of electronic supplementary material.

A polymorphism in the gene coding for triose phosphate isomerase (TPI) which plays an important role in glycolysis and is essential for efficient energy production is also shown in table 5 of electronic supplementary material. TPI deficiency leads to a metabolic block of the glycolytic pathway and hence a generalized impairment of cellular energy supply causing generalized skeletal muscle deficiency.

Insulin-like growth factors and related proteins

Insulin-like growth factors (IGFs) are polypeptides with high sequence similarity to insulin. IGFs are part of a complex system that cells use to communicate with their physiologic environment. This complex system (often referred to as the IGF 'axis') consists of two ligands (IGF-1 and IGF-2), two cell-surface receptors (IGF1R and IGF2R), and a family of six high-affinity IGF-binding proteins (IGFBP1–IGFBP6) (Jones and Clemmons 1995). This system regulates normal cellular metabolism, proliferation, differentiation and protecting against apoptotic signals (Jerome *et al.* 2003). High levels of IGF-1 and IGFBP3 have been implicated with poorer prognosis in certain types of cancers. Polymorphisms in genes coding for components of the IGF axis have been shown to affect serum levels of their respective proteins as well as body composition (see table 6 in electronic supplementary material).

Also shown in table 6 of electronic supplementary material are polymorphisms in the iodothyronine deiodinase, type 1 gene (DIO1) which activates thyroid hormone by converting the prohormone thyroxine (T4) by outer ring deiodination to bioactive triiodothyronine (T3). Thyroid hormone is known to interact with the GH-IGF-1 axis although the exact mechanism is unknown (Peeters *et al.* 2005).

Corticosteroid signalling proteins

Corticosteroids are essential steroid hormones that are secreted by the adrenal cortex and affect multiple organ systems. Corticosteroids are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation, carbohydrate metabolism and protein catabolism. The genetic variants of the components in the mechanism of corticosteroid signalling are examined in table 7 of electronic supplementary material.

Muscle

Muscle atrophy is known to occur in cancer cachexia. This results from the depression of muscle protein synthesis, an increase in muscle protein degradation, or a combination of both (Eley and Tisdale 2007). Experimental studies have also shown a shift in the myosin isoform content of skeletal muscle in cancer cachexia from type I to type II (Diffee *et al.* 2002). The following section examines the genetic variations that affect the structure and function of muscle as well as those that regulate muscle proteolysis.

Muscle structure and function

Genes involved in regulating muscle structure and function include those coding for ACTN3 (Alpha-actinin 3), which bind to actin at the Z-line within muscle fibres and act to anchor actin filaments, and IL15 (interleukin-15). IL-15 signals through IL-15 receptor alpha (IL-15RA) and is found in abundance in skeletal muscle. IL-15 is shown to be anabolic, marked by an increase in myosin heavy chain accumulation (Quinn *et al.* 2002). Polymorphisms in ACTN3, IL15 and IL15RA are shown in table 8 of electronic supplementary material.

Steroid androgens play an important role in determining LBM and muscle strength. Variants in the gene coding for the androgen receptor (AR), which is activated by binding of either androgenic hormones testosterone or dihydrotestosterone and are known to be capable of activating myogenic genes (Vlahopoulos *et al.* 2005), are also displayed in table 8 of electronic supplementary material. Polymorphisms associated with alterations fat free mass in the gene encoding the vitamin D receptor (VDR) are also included in table 8 of electronic supplementary material.

Muscle proteolysis

Angiotensin converting enzyme (ACE) plays a critical role in the renin-angiotensin system by catalysing the conversion of the inactive angiotensin I to angiotensin II, which is the physiologically active form of the hormone. Acute and chronic exposure to angiotensin II in animal models are associated with weight loss and enhanced protein breakdown in skeletal muscle (Brink *et al.* 1996, 2001).

In atrophying muscles, the ubiquitin ligase, atrogin-1, is induced and this response is necessary for rapid atrophy. FOXO3 is known to act on atrogin-1 promoter to cause atrogin-1 transcription and this leads to dramatic atrophy of myotubes and muscle fibres (Sandri *et al.* 2004). Polymorphisms in *ACE* and *FOXO3A* genes are presented in table 9 of electronic supplementary material.

Adipose tissue

Body-fat depletion is a major component of weight loss in cancer cachexia. Increased lipolysis appears to be a key factor underlying fat loss, though decreases in lipid deposition and adipocyte development may also contribute (Legaspi *et al.* 1987). The following section examines polymorphisms in genes regulating adipose tissue metabolism.

Adipogenesis

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. There are three subtypes of the receptor (PPARα, PPARβ and PPARγ). PPARα is most commonly expressed in organs

and tissues in which fatty acid oxidation is active. PPARα is known to participate in the regulation of key proteins involved in extracellular lipid metabolism, fatty acid oxidation and inflammation (Torra *et al.* 2001). PPARγ is an important regulator of fat cell function by orchestrating differentiation of new adipocytes and by inducing expression of genes promoting uptake of fatty acids, triglyceride synthesis and insulin sensitivity (Lehrke and Lazar 2005). Polymophisms in the *PPARA* and *PPARG* genes are shown in table 10 of electronic supplementary material.

Also of interest are the lipin proteins (lipin-1, lipin-2 and lipin-3) which are thought to be required for glycerolipid biosynthesis. They also act as transcriptional coactivators that regulate expression of lipid metabolism genes (Reue 2009). Variants of genes coding of lipin-1 (LPIN1) and lipin-2 (LPIN2) are also presented in table 10 of electronic supplementary material.

Lipid turnover and transport

During lipolysis, trigylcerides are broken down in a stepwise fashion to free fatty acids (FFAs). This process is partly modulated by the sympathetic nervous system-induced secretion of cathecholamines which act through β1, β2 and β3 adrenergic receptors (β-ARs) (Large *et al.* 2004). Signals between β-adrenergic receptors and effector proteins are integrated by G proteins which are composed of alpha, beta, and gamma subunits. Polymorphisms of genes coding for β-ARs and the beta subunits of the G proteins (GNB3) are shown in table 11 of electronic supplementary material.

Lipoprotein lipase (LPL) is an enzyme that hydrolyses lipids in lipoproteins and plays a central role in the overall lipid metabolism and transport (Mead *et al.* 2002). Fatty acid binding proteins (FABPs) are a family of carrier proteins for fatty acids and other lipophilic substances. These proteins are thought to facilitate the transfer of fatty acids between extracellular and intra-cellular membranes (Chmurzynska 2006). Polymorphisms in the *LPL* and *FABP* genes are shown in table 11 of electronic supplementary material.

Adiopkines and adipokine receptors

Adipose tissue is also recognized as a major endocrine organ, because the tissue synthesizes and secretes an array of protein hormones and signals (Fantuzzi 2005). These adipokines act locally in an autocrine/paracrine manner and/or as endocrine signals to regulate appetite, energy expenditure and a range of physiological processes including insulin sensitivity and inflammatory response which may have an important role in the pathogenesis of cancer cachexia (Kerem *et al.* 2008).

Resistin is an adipocyte-derived proinflammatory cytokine. Resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al.* 2006). There are three polymorphisms within the *RETN* gene which codes for resistin that may influence the development of cachexia (see table 12 in electronic supplementary material).

Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (Diez and Iglesias 2003). Adiponectin binds to a number of receptors including adiponectin receptors 1 and 2. An increase in adiponectin concentration has been assoicated with cachexia in patients with heart failure (McEntegart *et al.* 2007). Leptin is a protein hormone secreted by adipose tissue that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. Leptin acts through the leptin receptor. Polymorphisms in genes coding for adiponectin, leptin and their respective receptors are given in table 12 of electronic supplementary material.

Appetite regulation

Anorexia, defined as the loss of desire to eat, is common in patients with cancer cachexia. In addition to any effects of the tumour on the gastrointestinal tract and psychological depression, patients with cancer frequently have a decreased taste and smell of food (DeWys and Walters 1975). Cancer anorexia may be a result of an imbalance between orexigenic signals and anorexigenic signals. The following section explores the variants in the genes encoding these signals.

Ghrelin is synthesized principally in the stomach and is released in response to acute and chronic changes in nutritional state. In addition to having a powerful effect on the secretion of growth hormone, ghrelin stimulates food intake and transduces signals to hypothalamic regulatory nuclei that control energy homeostasis (Hosoda *et al.* 2002). The melanocortin 4 receptor, a G-protein coupled receptor, binds α-melanocyte stimulating hormone (α-MSH). Melanocortin 4 receptors have been found to be involved in feeding behaviour and the regulation of metabolism (Fan *et al.* 1997). Endocannabinoids, acting at brain cannabinoid type 1 receptors, play a role in stimulating appetite and ingestive behaviours, partly through interactions with more established orexigenic and anorexigenic signals (Kirkham 2005). Ciliary neurotrophic factor (CNTF) has been shown to activate hypothalamic leptin-like pathways which suppress food intake without triggering hunger signals or associated stress responses that are otherwise associated with food deprivation (Lambert *et al.* 2001). CNTF acts via CNTF receptors (CNTFRs) which are located in hypothalamic nuclei involved in feeding. Polymorphisms of interest in the genes encoding ghrelin (GHRL), melanocortin 4 receptor (MC4R), cannabinoid type 1 receptor (CNR1) and the CNTFR are given in table 13 of electronic supplementary material.

Others

Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins. MTs are encoded by a family

of genes consisting of 10 functional MT isoforms, and the encoded proteins are conventionally subdivided into four groups: MT-1, MT-2, MT-3 and MT-4 proteins. The physiological roles of MTs are not well understood but they are thought to play a role in the control of oxidative stress and protection against inflammation (Simpkins 2000).

The P2Y-receptors belong to the superfamily of G-protein-coupled receptors and mediate the actions of extracellular nucleotides in cell-to-cell signalling. The P2Y11 receptor is highly expressed in immunocytes and may play a role in the differentiation of these cells (von Kugelgen 2006).

Glutamine : fructose-6-phosphate aminotransferase (GFAT) is the rate-limiting enzyme of the hexosamine biosynthetic pathway (HBP), which catalyses the conversion of fructose-6-phosphate to glucosamine-6-phosphate. The flux through the HBP has been shown to be linked to the regulation of energy intake and energy expenditure (Obici *et al.* 2002).

Fat mass and obesity associated (FTO) is thought to play a role in energy homeostasis but its exact function is unknown.

Genetic variants encoding the proteins discussed above may play a role in the development of cachexia and are listed in table 15 of electronic supplementary material.

Analysis of results

Out of 184 polymorphisms that have been identified, the functional or clinical significance of only 42 polymorphisms have been verified in more than one study.

Of these 42 polymorphisms, 13 have been shown to have more than one effect on clinical features associated with cancer cachexia (i.e. inflammation, changes in lean and/or fat mass, and overall survival). These 13 polymorphisms represent the most promising candidates in terms of susceptibility biomarkers of cancer cachexia (table 1) and are explored in more detail below.

The C allele of the A37674C *SELP* polymorphism (rs6136) is associated with decreased serum P-selectin levels (Miller *et al.* 2004; Volcik *et al.* 2006). P-selectin is required for efficient recruitment of neutrophils in acute inflammation and of macrophages in later stages of the inflammatory response and serum levels of P-selectin have been found to be significant prognostic factors in survival in patients with gastric and colorectal malignancies (Alexiou *et al.* 2001, 2003)

TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Within the *TNF* gene, the –308A allele (rs1800629) has been associated with an increased TNF-α production as well as a 6-fold increase in transcription of the *TNF* gene (Wilson *et al.* 1997; Sallakci *et al.* 2005). Interestingly, the A/A genotype has been linked to increased fat accumulation in women (Hoffstedt *et al.* 2000). The -863A allele (rs1800630) associated with decreased transcriptional activity and reduced serum TNF-α levels (Day *et al.* 1998; Skoog *et al.* 1999; Kaluza *et al.* 2000; Sharma *et al.* 2006). Of note, obese individuals express 2.5-fold more TNF mRNA in fat tissue (Hotamisligil *et al.* 1995).

LTA, a member of the tumour necrosis factor family, is a cytokine produced by lymphocytes, and mediates a large variety of inflammatory and immunostimulatory, responses. The G allele of the 252 A>G polymorphism (rs909253) has been associated with increased serum TNF-α levels (Stuber *et al.* 1996; McArthur *et al.* 2002), and patients who are A/A homozygotes have been linked with better prognosis in lung cancer and gastric cancer (Shimura *et al.* 1994, 1995).

IL-1β is a cytokine protein which is encoded by the *IL1B* gene and is an important mediator of the inflammatory response. The -31 C>T (rs1143627) and -511 C>T (rs16944) polymorphisms in the promoter region of the *IL1B* gene have been linked with increased transcriptional activity of the *IL1B* gene and subsequently increased IL-1β production (Wen *et al.* 2006). The -31C and -511T alleles are linked with poorer progression-free survival and overall survival in advanced gastric cancer (Graziano *et al.* 2005). Increased IL-1β levels have been linked to a synonymous C to T polymorphism at nucleotide position 3953 (rs1143634) (Hernandez-Guerrero *et al.* 2003). The T/T genotype has also been associated with lower plasma levels of IL-1 receptor antagonist (IL-1RA) (Tolusso *et al.* 2006). In addition, the T allele has found to be a major risk factor for cachexia in gastric cancer (Zhang *et al.* 2007), as well as being linked to lower total fat mass (Strandberg *et al.* 2006). The T/T genotype was found to be associated with shorter survival in pancreatic cancer (Barber *et al.* 2000).

IL-6 is a cytokine involved in a wide variety of biological functions. It is critical for B-cell differentiation and maturation, immunoglobulin secretion, cytotoxic T-cell differentiation and acute-phase protein production (Kishimoto 2005). The -174 G>C promoter polymorphism (rs1800795) in the *IL6* gene has been associated with lower serum levels of IL-6 (Fishman *et al.* 1998). The G allele has been linked to higher fasting insulin and lower adiponectin levels which may have a role in the regulation of adiposity (Yang *et al.* 2005). In addition the C/C genotype has been associated with lower fat free mass and increased waist circumference (Berthier *et al.* 2003; Roth *et al.* 2003).

IGF-1 plays an important role in childhood growth and continues to have anabolic effects in adults. IGF-1 is one of the most potent natural activators of the Akt signalling pathway, a stimulator of cell growth and multiplication. IGF-1 also mediates many of the growth-promoting effects of growth hormone (GH) (Jones and Clemmons 1995). A CA repeat polymorphism is found within the promoter region of the *IGF1* gene. The 19CA repeat allele is associated with both lower serum IGF-I levels and IGFBP-3 levels (Rosen *et al.* 1998; Morimoto *et al.* 2005). The 19CA repeat allele is also associated with reduced risk of weight gain (Landmann *et al.* 2006; Voorhoeve *et al.* 2006). Of note, increasing IGF-1 levels have been associated with poorer prognosis in oesophageal cancer patients (Sohda *et al.* 2004).

Table 1. Polymorphisms replicated in more than one study and with at least two effects on clinical features associated with cancer cachexia ($n = 13$).

Gene	Polymorphism	Minor allele frequency	Functional significance	Systemic inflammation	BMI/fat mass	Lean mass /strength	Cancer survival
SELP	A37674C (715 Thr→Pro) [rs6136]	9%	Decreased serum P-selectin levels (Miller *et al.* 2004; Volcik *et al.* 2006)	↓			↑
TNF	G-308A [rs1800629]	17%	Increased *TNF-α* production (Sallakci *et al.* 2005) Six-fold increase in transcription of *TNF-α* (Wilson *et al.* 1997)	↑	↑		
	C-863A [rs1800630]	15%	Reduced total serum IgE levels (Sharma *et al.* 2006) Reduced serum *TNF-α* levels (Sharma *et al.* 2006) 31% decrease in transcription of *TNF-α* (Skoog *et al.* 1999)	↓	↓		
LTA	A252G (Intron 1) [rs909253]	36%	Increased serum *TNF-α* levels (Stuber *et al.* 1996; McArthur *et al.* 2002)	↑			↓
IL-1β	C-31T [rs1143627]	36%	Increased expression of *IL-1β* gene with T allele (Lind *et al.* 2007)				
			Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al.* 2006)	↑			↑
			Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al.* 2006)				
	C-511T [rs16944]	36%	Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al.* 2006)				
			Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al.* 2006)	↓			↓
			No significant increase in IL-1β production in response to LPS in patients homozygous for T allele (Awomoyi *et al.* 2005)				
IL-1β	C3953T (3954) (synonymous) [rs1143634]	21%	T/T genotype associated with lower plasma levels of IL1-RA (Tolusso *et al.* 2006)	↑	↓		↓
			Increased human amniochorion IL-1β production after stimulation with LPS (Hernandez-Guerrero *et al.* 2003)				
IL6	G-174C [rs1800795]	46%	Lower levels of IL-6 in plasma in healthy subjects (Fishman *et al.* 1998)				
			Higher fasting plasma insulin levels with G allele (Yang *et al.* 2005) Lower circulating adiponectin levels with G allele (Yang *et al.* 2005)	↓	↑	↓	
IGF1	CA repeat promoter polymorphism 19 CA repeats (192bp)	?	Lower serum IGF-I levels (Rosen *et al.* 1998; Morimoto *et al.* 2005)		↓		↑
			Lower IGFBP-3 levels (Morimoto *et al.* 2005)				
ACE	Insertion/deletion (I/D) polymorphism Intron 16 (287bp) [rs4646994]	?	I allele associated with lower ACE levels (Tiret *et al.* 1992)		↑	↑	↑
LPL	447 Ser→TER [rs328]	12%	Significantly lower IL-8 levels (Ak *et al.* 2007) Increased LPL activity (Kozaki *et al.* 1993; Groenemeijer *et al.* 1997)	↓	↓		
RETN	C-420G [rs1862513]	35%	Increased plasma resistin (Cho *et al.* 2004; Osawa *et al.* 2007)	↑	↑		
ADIPOQ	T45G (synonymous) [rs2241766]		Increased plasma adiponectin (Berthier *et al.* 2005; Mackevics *et al.* 2006)		↓	↑	

↑ Increase; ↓ decrease; ?, allele frequency unknown.

Angiotensin converting enzyme (ACE) plays a critical role in the renin-angiotensin system by catalysing the conversion of the inactive angiotensin I to angiotensin II, which is the physiologically active form of the hormone. Acute and chronic exposure to angiotensin II in animal models are associated with weight loss and enhanced protein breakdown in skeletal muscle (Brink *et al.* 1996, 2001). A common insertion/deletion (I/D) polymorphism (rs4646994) is present in intron 16 of the *ACE* gene. The I allele is associated with lower circulating ACE levels (Tiret *et al.* 1992). The D allele is associated with increased strength gains thorough isometric training (Folland *et al.* 2000) and also associated with obesity (El-Hazmi and Warsy 2003; Riera-Fortuny *et al.* 2005). The D/D genotype is linked with increased survival in women with colorectal cancer (Rocken *et al.* 2007).

Lipoprotein lipase (LPL) is an enzyme that hydrolysis lipids in lipoproteins and plays a central role in the overall lipid metabolism and transport (Mead *et al.* 2002). The rs328 polymorphism in the *LPL* gene results in a premature stop codon at amino acid 447. The stop codon results in lower LPL activity (Kozaki *et al.* 1993; Groenemeijer *et al.* 1997), and is associated with lower levels of IL-8 (Ak *et al.* 2007). Individuals not in possession of the stop codon are associated with central obesity (Huang *et al.* 2006).

Resistin is an adipocyte-derived pro-inflammatory cytokine. Resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al.* 2006). The –420 C>G polymorphism (rs1862513) is shown to be linked to increased plasma resistin (Cho *et al.* 2004; Osawa *et al.* 2007), and individuals with the G/G genotype are associated with an increased prevalence of obesity (Norata *et al.* 2007). Overall, increased plasma resistin has to shown to correlate with increased CRP and insulin resistance (Degawa-Yamauchi *et al.* 2003; Silswal *et al.* 2005; Nagaev *et al.* 2006; Kusminski *et al.* 2007; Osawa *et al.* 2007).

Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (Diez and Iglesias 2003). An increase in adiponectin concentration is associated with cachexia in patients with heart failure (McEntegart *et al.* 2007). The *ADIPOQ* gene, which codes for adiponectin, has a 45 T>G polymorphism (rs2241766) that is associated with increased plasma adiponectin (Berthier *et al.* 2005; Mackevics *et al.* 2006). Individuals with G/G genotype have been observed to be leaner with less abdominal fat (Loos *et al.* 2007).

Pathway analysis

The 42 polymorphisms which have been verified in more than one study were found across 33 genes. These genes were entered into the IPA algorithm as focus genes and were found to be significantly interconnected in two major networks (table 2). Using the 'overlapping network' feature of IPA, we found that these networks were joined together by few genes, namely *ADIPOQ, IL6, NFKB1 and TLR4*. The two networks are presented in figures 1 and 2.

Discussion

Support for a heritable component to cachexia has recently been highlighted in several studies. The IL6 -634G allele is associated with increased susceptibility to cachexia and decreased survival time of Chinese patients suffering from pancreatic cancer (Zhang *et al.* 2008). Another study by

Table 2. Ingenuity pathway analysis of genes that were replicated in more than one study ($n = 33$).

Network	Molecules*	Calculated score	Focus genes	Top functions of network
1	**ADIPOQ**, Akt, Ap1, **CCL2**, ERK, ERK1/2, Gm-csf, histone h3, histone h4, IFN beta, **IFNG**, **IGF1**,**IGFBP3**, IgG, **IL6**, IL12 (complex), **IL1B**, immunoglobulin, interferon alpha, Jnk, LDL, **LEP**, Mapk,**NFKB1**, NFkB (complex), P38 MAPK, PDGF BB, PI3K, **PPARG**, **RETN**, Sod, **TLR2**,**TLR4**,**TNF**, Vegf	24	14	Connective tissue disorders, inflammatory disease, skeletal and muscular disorders
2	**ADIPOQ**, **ADRB2**, BIRC3, BTG2, CAMP, CREBBP, **CRP**, CSF1, F2, GSK3B, HDAC3, HGF, HMGB1 (includes EG:3146), HSPD1, IKBKB, **IL6**, IL17A (includes EG:3605), IL6R, IRS1, **LPL**, LTF, NFIC, **NFKB1**, NOS2, **NR3C1**, OSM, PLD1, PLG, RELA, **SELP**, SIRT1, **TLR4**, TNFSF12, TP53,TSC22D3	13	9	Skeletal and muscular system development and function, cell death, cellular movement

*Focus genes are in bold.

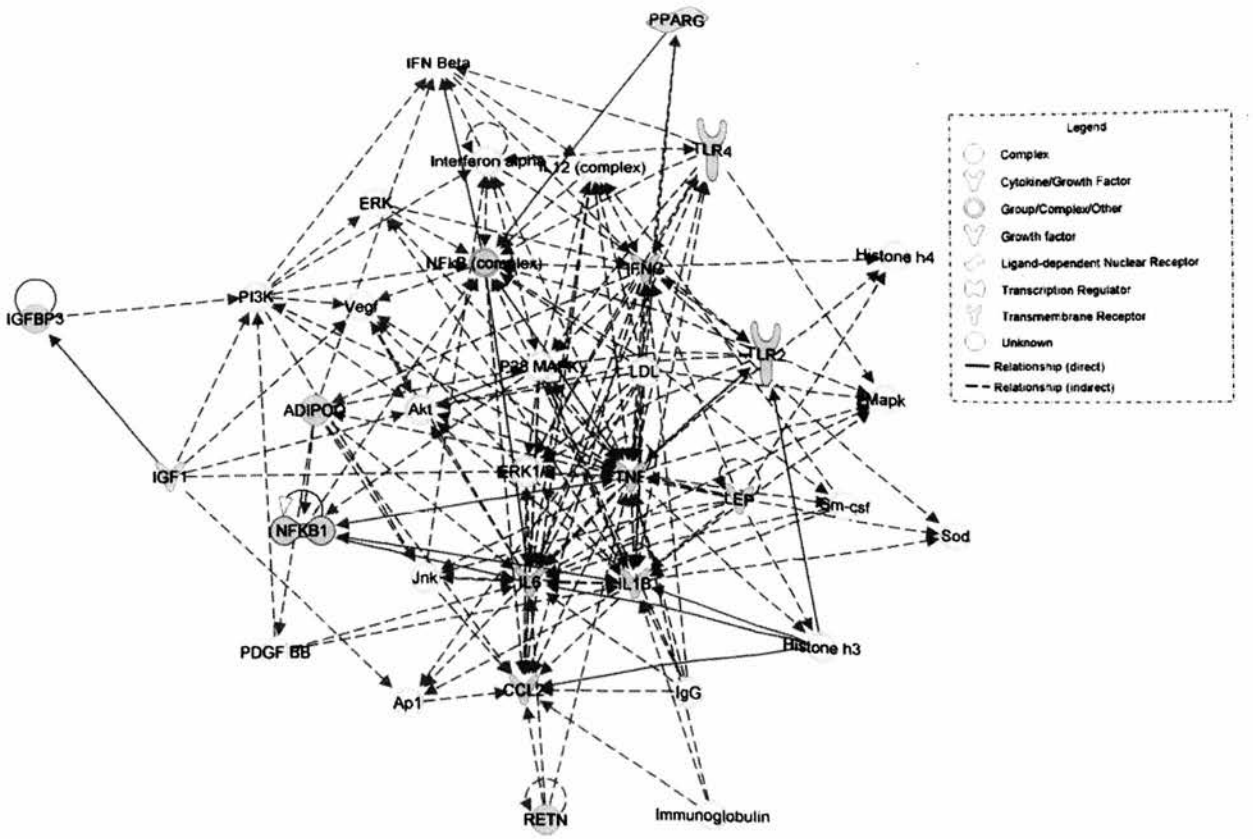

Figure 1. Connection map for first ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervised functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥ 1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

Deans *et al.* (2009) revealed that the IL10 -1082GG genotype was associated with 2.3 times increased risk of developing cachexia in patients with gastroesophageal malignancy.

However, like many complex conditions and diseases, the risk of developing cancer cachexia is probably determined by multiple genetic factors and environmental factors are likely to add to the heterogeneity of the condition. Although the number of reports on polymorphic gene variants associated with multi-factorial diseases and conditions are dramatically growing, very few studies provide firm and reliable evidence of causative relationships between these polymorphisms and risk or pathogenesis. Indeed, in a recent review of allelic association with common disease phenotypes, only six of the 166 associations subjected to multiple evaluations were confirmed consistently (Hirschhorn *et al.* 2002). Possible causes of false-positive association studies include population stratification, variable linkage disequilibrium and genotype misclassification. In addition, in many of these studies, the possible effects of single gene variants were assessed in situations when combined impacts of multiple factors could be expected (Loktionov 2003). In the present study, we have identified 42 polymorphisms out of 184 with a potential role in the development of cachexia that have been independently verified in at least one repeat study.

It can be assumed that analysis of combinations of gene variants encoding interacting factors within a biological chain or cascade, rather than isolated investigation of its single components, may have more chances to reveal real causative connections between gene polymorphisms and phenotypes. In this study, functional polymorphisms in genes with a possible role in cachexia have been recorded as well as polymorphisms with clinical consequences related to cachexia such as inflammation, weight/body composition changes and cancer survival.

Of the 42 polymorphisms with a potential role in the development of cachexia that have been independently verified in at least one repeat study, 13 polymorphisms have been shown to have more than one effect on clinical features associated with cancer cachexia. These 13 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers of cancer cachexia and should be further investigated.

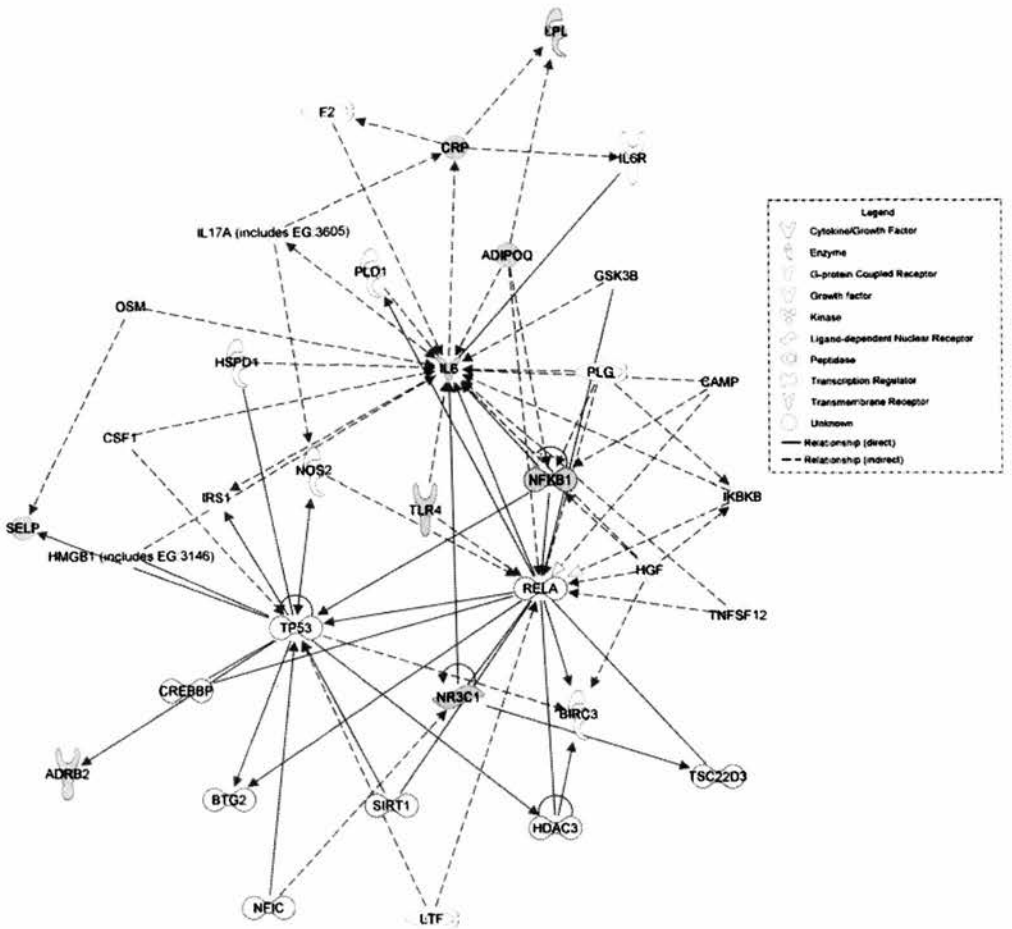

Figure 2. Connection map for second ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervised functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥ 1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

Pathway analysis of the independently verified genes has revealed four genes interlinking two putative major networks involved in the development of cancer cachexia. These four genes (*ADIPOQ, IL6, NFKB1* and *TLR4*) may have central roles in the pathogenesis of cancer cachexia and should be further investigated.

It should also be emphasized that while certain variant combinations can predispose to a condition, others may be protective against the same condition. For example, polymorphisms resulting in an increase in systemic inflammation, a decrease in lean or fat mass and decreased survival in cancer are likely to be cachexia prone variants. On the other hand, polymorphisms that result in a decrease in systemic inflammation, an increase in body mass and improved survival in cancer are likely to be cachexia resistant variants.

In multigene studies, judicious selection of candidate genes and polymorphisms within them is a key element of study design. It is always important to choose genes, products of which interact within regulatory or metabolic pathways. In most cases, it is not realistic to analyse all possible gene variants and combinations, hence existing polymorphisms should be initially prioritized on the basis of their likelihood to affect function of the encoded product (Tabor *et al.* 2002).

The present study has provided an initial framework to select and study the possible genetic variance in developing cancer cachexia by identifying polymorphisms with putative functional and/or clinical significance in relation to the development of cachexia. The identification of genetic variants that have undergone repeat studies allows the selection of robust and reliable candidates. Furthermore, prioritization of the most likely genetic variants that are likely to influence the development of cachexia have been derived by selecting polymorphisms that influence two or more hallmarks of cachexia (i.e. systemic inflammation, loss of fat mass, loss of lean mass and reduced survival).

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P-selectin genotype is associated with the development of cancer cachexia

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The variable predisposition to cachexia may, in part, be due to the interaction of host genotype. We analyzed 129 single nucleotide polymorphisms (SNPs) in 80 genes for association with cachexia based on degree of weight loss (>5, >10, >15%) as well as weight loss in the presence of systemic inflammation (C-reactive protein, >10 mg/l). 775 cancer patients were studied with a validation association study performed on an independently recruited cohort ($n = 101$) of cancer patients. The C allele (minor allele frequency 10.7%) of the rs6136 (*SELP*) SNP was found to be associated with weight loss >10% both in the discovery study (odds ratio (OR) 0.52; 95% confidence intervals (CI), 0.29–0.93; $p = 0.026$) and the validation study (OR 0.09, 95% CI 0.01–0.98, $p = 0.035$). In separate studies, induction of muscle atrophy gene expression was investigated using qPCR following either tumour-induced cachexia in rats or intra-peritoneal injection of lipopolysaccharide in mice. P-selectin was found to be significantly upregulated in muscle in both models. Identification of P-selectin as relevant in both animal models and in cachectic cancer patients supports this as a risk factor/potential mediator in cachexia.

INTRODUCTION

Cachexia is a wasting condition that manifests itself in several life-threatening diseases, including cancer, AIDS, congestive heart failure and sepsis (Argiles et al, 2003; Tisdale, 2004). Patients exhibit a loss of both adipose tissue and lean body mass (Fearon & Preston, 1990), which is resistant to conventional nutritional support (Tisdale et al, 1987). Cachexia is typically characterized by severe weight loss, anorexia, early satiety, weakness, anaemia and oedema (Fearon & Preston, 1990). The cachectic state is particularly problematic in cancer, typified by poor prognosis and often associated with a lower response to chemotherapy and radiotherapy than might be expected (Tisdale, 2002). Patients are also more likely to report decreased quality of life (QoL) scores (Fearon et al, 2006). More than half of cancer patients suffer from cachexia, and it is responsible for death in up to 20% of cases (Tisdale, 2002). Cachexia is therefore a significant cause of morbidity and mortality in cancer patients.

Based on our current knowledge of demographic and clinical factors, we are unable to predict, for any given cohort of

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patients, who will develop cancer cachexia and who will not. Such variation may, in part, be due to the patient's genotype. Knowledge of genotypic variation associated with cachexia would contribute to early identification of patients at risk and allow institution of prophylactic measures. The wealth of known genetic polymorphisms in genes controlling pro/anti-inflammatory pathways, neuronal melanocortin signalling pathways and muscle and adipose tissue catabolic pathways suggest their exploitable potential as biomarkers of inter-individual predictability of developing cachexia.

We utilized a candidate gene approach to evaluate the association between genetic polymorphisms and the risks of developing cachexia in patients recruited across three centres. Patients recruited from a fourth centre were used as a validation cohort. To further corroborate the most significantly related single nucleotide polymorphism (SNP) to cancer cachexia in the gene association study, we tested the same gene for participation in the induction of the skeletal muscle atrophy gene program, either by intra-peritoneal administration of lipopolysaccharide (LPS) in mice or in a rat model of cancer cachexia (methylcholanthrene (MCA)-induced sarcoma). LPS is known to induce acutely a number of catabolic factors in sepsis, suppress anabolic factors and result in muscle atrophy (Dehoux et al, 2003; Vary et al, 1998). The MCA model is a preclinical cancer cachexia model, and is known to reliably induce loss of lean body mass (Sato et al, 2001).

Table 1. Patient demographics (main cohort). Patients were recruited from (2004 to 2008) from the NHS Lothian, UK, Cross Cancer Institute, Edmonton, Canada, and McGill University Health Centre, Montreal, Canada

	No. of patients ($n = 775$)
Age (years) †	65.5 ± 11.8
Range	27–97
Sex	
M	476 (61.4)
F	299 (38.6)
Tumour type	
Oesophageal or gastric	389 (50.2)
Pancreatic	114 (14.7)
Non-small cell lung cancer	232 (29.9)
Other	40 (5.2)
Stage	
I	38 (4.9)
II	95 (12.3)
III	216 (27.9)
IV	392 (50.5)
Unknown	34 (4.4)
Body mass index (kg/m^2) †	24.9 ± 4.9
Range	12.9–46.7
Percentage weight loss †	7.95 ± 8.16
Range	0–43.8
C-reactive protein (mg/l)† ($n = 569$)	23.0 ± 35.9
CRP > 10 mg/l	235 (41.3)
CRP ≤ 10 mg/l	334 (58.7)

Values are number of patients with percentages in parentheses unless indicated otherwise.
†values are mean ± SD. Characteristics were measured at first presentation to a surgical or oncology clinic.

RESULTS

Following the relevant quality control checks, 129 SNPs in 80 genes (Supporting Information Table S1) were available for analysis in 775 patients. The overall completion rate of genotyping was 95.6%.

The general characteristics of the study population are presented in Table 1. Average age of the patient cohort at diagnosis was 65.5 ± 11.8 years (mean ± SD). The majority of patients were diagnosed with stage III or IV cancers. Average weight loss was 6.9 ± 9.8% with a mean body mass index (BMI) of 24.9 ± 4.9 at diagnosis. Of the patients in whom C-reactive protein (CRP) levels were assessed ($n = 569$), 58.7% had a CRP concentration of >10 mg/l. There were no significant differences in age, stage of disease, pre-diagnosis BMI and percentage weight loss between patients with CRP measured and the entire cohort.

Table 2a lists the detailed results for SNPs significantly associated with cancer cachexia in patients classified according to weight loss alone. Table 2b lists the detailed results for SNPs significantly associated with cancer cachexia in patients classified according to weight loss with systemic inflammation (CRP >10 mg/l). In total, eight SNPs have associations of $p < 0.02$ with various cachexia phenotypes. Three of these SNPs are found within chromosome 1 in the genes selectin P (*SELP*), leptin receptor (*LEPR*) and deiodinase, iodothyronine, type I (*DIO1*); three within chromosome 3 in the genes N-acylaminoacyl-peptide hydrolase (*APEH*) and ghrelin (*GHRL*), one within chromosome 12 in the *TNFRSF1A* gene and one within chromosome 19 in the *ICAM1* gene. SNPs found on the same chromosomal region (within 10 000 kb) were grouped together to form haplotypes. The haplotypes formed by the rs4855881 and rs2960548 SNPs in the *APEH* gene failed to show any significant association with weight loss.

Analyses of candidate gene groups based on functional similarity revealed three groups that were associated with at least one cachexia phenotype at the $p < 0.05$ level (Table 3).

Validation study

Patient demographics of the validation cohort ($n = 101$) are presented in Table 4. Although, patients in the validation cohort did not have an identical distribution of cancer types as the main cohort, the distribution of BMI and weight loss remain quite similar between the two cohorts. Approximately 60% of the patients in the validation cohort had other cancer types which also had tendency to develop cachexia like prostate cancer and colorectal cancer (it is estimated that 30% of patients suffering from these cancers have a weight loss of 5% or more (Dewys et al, 1980)).

Study subjects were genotyped for SNPs with $p < 0.05$ in the main study. One replication of the main study was found. The C allele of the rs6136 SNP was inversely associated with weight loss >10% in the main study (odds ratio, OR 0.52; 95% confidence intervals, 95% CI 0.29–0.93; $p = 0.026$) as well as in the validation study (OR 0.09, 95% CI 0.01–0.98, $p = 0.035$).

Table 2a. Genes with variants significantly associated with cancer cachexia in patients classified according to weight loss alone

Weight loss >15%. Number affected: 145/775 (18.7%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
SELP	rs6136	C	0.31 (0.14–0.72)	0.006615	0.008062
ICAM1	rs281432	G	1.53 (1.06–2.20)	0.02163	0.01652
DIO1	rs11206244	T	1.54 (1.06–2.24)	0.0226	0.02164
ADIPOR2	rs16928751	A	0.53 (0.29–0.96)	0.03521	0.03053
APEH	rs2960548	G	1.48 (1.03–2.11)	0.03384	0.03768

Weight loss >10%. Number affected: 266/775 (34.3%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
LEPR	rs1137100	G	0.66 (0.47–0.92)	0.01494	0.013
DIO1	rs11206244	T	1.52 (1.09–2.11)	0.0129	0.01512
SELP	rs6136	C	0.52 (0.29–0.93)	0.02746	0.02581
HYLS1	rs3088241	C	0.72 (0.53–0.97)	0.02829	0.02709
CAMK2B	rs10441113	A	0.73 (0.54–0.99)	0.04096	0.03419

Weight loss >5%. Number affected: 415/775 (53.5%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
TNFRSF1A	rs4149570	T	1.42 (1.08–1.87)	0.01134	0.01759
TNFRSF1A	rs767455	C	0.71 (0.53–0.95)	0.02034	0.02275
TNFRSF1B	rs976881	A	0.76 (0.57–1.00)	0.04804	0.04324
IL18	rs1946519	A	1.35 (1.02–1.79)	0.03895	0.04969

Table 2b. Genes with variants significantly associated with cancer cachexia in patients classified according to weight loss with systemic inflammation (CRP >10 mg/l)

Weight loss >15% & CRP >10 mg/l. Number affected: 76/569 (13.4%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
APEH	rs2960548	G	2.17 (1.36–3.47)	0.001125	0.000997
GHRL	rs42451	T	2.04 (1.25–3.31)	0.004031	0.004058
TNFRSF1A	rs4149570	T	1.84 (1.16–2.92)	0.009322	0.01031
SELP	rs6136	C	0.26 (0.08–0.79)	0.01765	0.01103
CNR1	rs1049353	A	1.82 (1.08–3.06)	0.02366	0.02254
IRS1	rs1025333	A	2.24 (1.07–4.69)	0.03257	0.03183
APEH	rs4855881	C	1.64 (1.04–2.59)	0.03431	0.03191
FOXO1	rs17446593	G	0.49 (0.26–0.92)	0.02704	0.03239
ICAM1	rs281432	G	1.63 (1.04–2.54)	0.03276	0.03941

Weight loss >10% & CRP >10 mg/l. Number affected: 123/569 (21.6%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
APEH	rs2960548	G	1.80 (1.21–2.68)	0.003528	0.003499
GHRL	rs42451	T	1.79 (1.18–2.72)	0.006219	0.00467
TNFRSF1A	rs4149570	T	1.51 (1.04–2.18)	0.02958	0.01998
HYLS1	rs3088241	C	0.66 (0.46–0.95)	0.02374	0.02074
APEH	rs4855881	C	1.57 (1.06–2.32)	0.02334	0.02847
TSC2	rs7187438	C	0.64 (0.43–0.95)	0.0265	0.03438
TNFRSF1B	rs3397	C	0.67 (0.46–0.97)	0.03527	0.04286

Weight loss >5% & CRP >10 mg/l. Number affected: 166/569 (29.2%)

Gene	SNP	Risk allele	OR (95% CI)	*p*-Value	Permutated *p*
APEH	rs2960548	G	1.67 (1.17–2.38)	0.004924	0.004533
APEH	rs4855881	C	1.56 (1.10–2.21)	0.01321	0.01212
TNFRSF1A	rs4149570	T	1.51 (1.08–2.10)	0.01559	0.02074
ADIPOR2	rs16928751	A	0.56 (0.33–0.95)	0.03308	0.02096
ADIPOR2	rs35854772	T	0.57 (0.33–0.97)	0.03733	0.02667
TNFRSF1B	rs3397	C	0.70 (0.50–0.98)	0.03944	0.02923
LTBP1	rs817529	G	0.70 (0.49–0.98)	0.03719	0.03791
TNFRSF1A	rs767455	C	0.68 (0.48–0.96)	0.02682	0.03846

Table 3. Candidate gene groups associated with cancer cachexia phenotypes

Phenotype	Candidate gene group function	Number of genes[†]	Number of SNPs	*p*-Values
Weight loss >10% & CRP >10 mg/l	Appetite regulation	2	3	0.0155
	Glucocorticoid signalling	4	9	0.0351
	MAPK activity regulation	7	14	0.0481
Weight loss >15% & CRP >10 mg/l	Appetite regulation	2	3	0.008499
	Glucocorticoid signalling	4	9	0.0181
	MAPK activity regulation	7	14	0.0264

[†]The genes in each candidate gene group are listed in Supporting Information Table S2.

Changes in skeletal muscle gene expression following either intra-peritoneal injection of LPS in mice or in rats bearing the MCA sarcoma

qPCR analysis of mouse skeletal muscle RNA performed after intra-peritoneal LPS injection revealed that the SELP (P-selectin) transcript was significantly differentially expressed compared with control (Fig 1a). In a separate study, rats with net loss of lean body mass and gastrocnemius mass due to growth of the MCA tumour (Fig 1c), showed similar upregulation of the SELP transcript. The latter was associated with significant upregulation of the 'atrogen' E3 ligases muscle atrophy F-box (MAFBx) and muscle ring finger 1 (MuRF1) along with forkhead box O1 (FOXO1), a transcription factor associated with muscle atrophy (Fig 1b).

Table 4. Patient demographics (validation cohort). Patients recruited from (2007 to 2008) from the Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland

	No. of patients ($n = 101$)
Age (years) [†]	62.0 ± 11.5
Range	35–88
Sex	
M	60 (59.4)
F	41 (40.6)
Tumour type	
Oesophageal or gastric	18 (17.8)
Pancreatic	6 (5.9)
Non-small cell lung cancer	19 (18.8)
Other	58 (57.4)
Stage	
I	0
II	3 (3.0)
III	2 (2.0)
IV	96 (95.0)
Body mass index (kg/m^2) [†]	23.7 ± 4.3
Range	15.4–37.8
Percentage weight loss [†]	5.54 ± 7.91
Range	0–43.1
C-reactive protein (mg/l)[†] ($n = 95$)	75.5 ± 76.4
CRP > 10 mg/l	78 (82.1)
CRP ≤ 10 mg/l	17 (17.9)

Values are number of patients with percentages in parentheses unless indicated otherwise.

[†]Values are mean ± SD. Characteristics were measured at first presentation to an oncology clinic.

DISCUSSION

This study has identified that individuals who carry the C-allele of the rs6136 polymorphism in *SELP* gene which encodes P-selectin, are at reduced risk of developing cachexia as defined by weight loss >10%. The C allele of the non-synonymous intronic variant, rs6136 has been previously associated with decreased serum P-selectin levels (Miller et al, 2004; Volcik et al, 2006). Information on P-selectin genotypes may eventually prove useful in the risk stratification of pre-cachectic cancer patients. Further evidence for the role of P-selectin in the development of cachexia is highlighted in the studies involving the induction of muscle atrophy in mice/rats. Strikingly, P-selectin was highly upregulated following either intra-

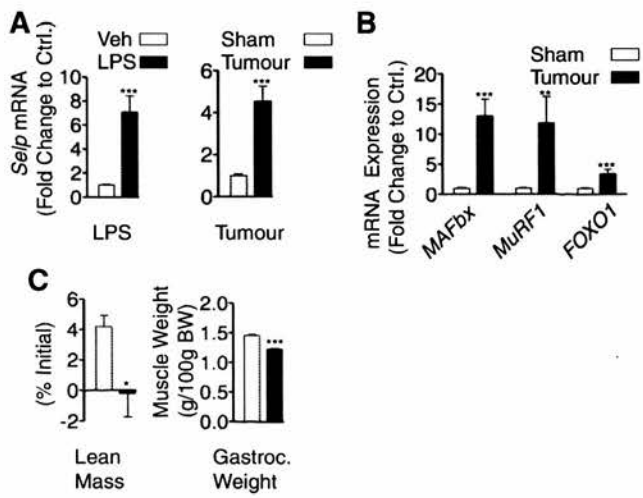

Figure 1. >Changes in skeletal muscle gene expression following either intra-peritoneal injection of LPS in mice or in rats bearing the MCA sarcoma. Wild type mice received either intra-peritoneal injections of LPS or vehicle alone. Food was removed from the cages at the time of injection, and animals were sacrificed at 8 h after the injection ($n = 6$–7/group).
Veh = vehicule. *Student's *t*-test $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A. Intra-peritoneal LPS treatment in mice or solid tumour growth in rats induces dynamic changes in P-selectin mRNA levels.

B. In tumour-bearing rats the changes in P-selectin expression are accompanied by concomitant upregulation of the E3-ligases (MAFBx and MuRF1) and transcription factor FOXO1.

C. The growth of the MCA sarcoma in rats is associated with net loss of lean body mass and muscle mass.

peritoneal injection of LPS or in tumour-bearing (TB) animals. Furthermore, preliminary studies also indicate that P-selectin show a similar striking upregulation (10-fold) 2 h after intra-cerebroventricular (ICV) injection of interleukin-1 beta (IL-1β) in mice (Braun et al, 2011). Acute and chronic infusion of IL-1β into the brain leads to muscle breakdown, anorexia, weight loss and negative nitrogen balance (Hill et al, 1996) and is a potential central mediator of LPS effects. Therefore, we have confirmed this gene target in three separate murine models of cachexia representing both acute and chronic inflammatory insults.

It could be argued that P-selectin expression in skeletal muscle is simply an endothelial event reflecting the presence of systemic inflammation. However, both identification of P-selectin as a top early induced gene of the mouse/rat muscle atrophy program and the significant association of the rs6136 SNP in the P-selectin gene with wasting in cancer patients provide supportive evidence for the likely involvement of P-selectin in muscle wasting. The role of P-selectin in the genesis of cachexia remains to be determined. The human P-selectin gene spans over 50 kbp on chromosome 1, containing 17 exons, almost all of which encode distinctive domain structures (Johnston et al, 1990; Watson et al, 1990). It has both membrane and soluble forms in platelets and endothelial cells (Johnston et al, 1990). Both the membrane and soluble forms of P-selectin bind to leukocytes. In certain inflammatory conditions, the plasma concentrations of soluble P-selectin is highly elevated (Dunlop et al, 1992). It is suggested, that the membrane and soluble forms of P-selectin may work co-ordinately *in vivo* for the regulation of their cell adhesion and, perhaps, signalling functionality. P-selectin has been characterized previously by approaches such as gene knockout or the use of specific inhibitors to be involved in the recruitment of neutrophils and macrophages in inflammatory responses (Borges et al, 1997; Chen & Geng, 2006). P-selectin may also participate in intra-tumoural regulation of the genesis of systemic inflammation via the innate immune system and/or regulation of the complex interaction within muscle between the endothelium and signalling pathways in muscle fibres (Wagenmakers et al, 2006).

CRP is a marker of systemic inflammation that has been studied in a wide variety of tumour types and has been linked to poorer survival (Mahmoud & Rivera, 2002; McMillan et al, 2003). To reflect that cachexia represents a spectrum and that the presence of systemic inflammation with weight loss may represent a unique sub-phenotype of cachexia which confers an increased mortality risk, we have chosen to study cachexia across three different percentage weight loss categories alone and with the presence of an increased CRP concentration in comparison with a weight-stable phenotype (*i.e.* $\leq$5% weight loss). Clearly, much work is required before fully validated definitions of cachexia are available. Until then, it appears reasonable to investigate cachexia based on the present definitions.

One limitation of the study is that patients were recruited at various stages of the disease process therefore there may be significant variation in time frame for weight loss. We have attempted to address this issue by adjusting the analyses for tumour stage at the time of recruitment assuming that patients who are diagnosed with more advanced disease would present with greater amount of weight loss. The amount of weight lost during the cancer journey may be affected by patients' pre-diagnosis BMI. The initially overweight/obese cancer patient may be more likely to lose a greater amount weight compared with a patient with the same cancer type in the normal BMI range over the same period of time. To account for this variation we have also adjusted the analyses for pre-diagnosis BMI. Another limitation of the study is that patients with upper GI malignancy often report dysphagia which may contribute to secondary malnutrition and influence the degree of weight loss. However, a previous study suggest that dysphagia may not be the sole contributing factor to weight loss in gastro-oesophageal malignancy as patients without dysphagia still report a median 4.4% weight loss at diagnosis. Moreover, in a multivariate model of the same cohort, dietary intake accounted for only 38% of variation in weight loss (Deans et al, 2009b).

The present study represents the first large scale candidate gene association study of cancer cachexia spanning a wide variety of genes such as genes that regulate inflammation, muscle and adipose tissue metabolism and appetite. SNPs chosen for the study were based on a literature review of SNPs with known functional effects and/or clinical relevance with regard to the development of cachexia (Tan et al, 2011). We also chose to analyze SNPs based on 18 genes identified in a gene expression study on muscle wasting in patients with cancer cachexia (Stephens et al, 2010). Instead of utilizing a tag SNP approach, as it was not realistic to analyze all possible gene variants and combinations, we selected SNPs that were most likely to be functional (*i.e.* within exons, non-synonymous and with a minor allele frequency (MAF) of >0.1) and hence more likely to be associated with the development of cachexia.

To further add strength to the study, we also attempted to validate the results by replicating the association study in an independently recruited group of patients. In the initial exploratory cohort we identified 21 SNPs in 17 genes with significant associations with cachexia phenotypes. However, when both the exploratory and validation cohorts were considered, only cancer patients carrying the minor allele (C) of rs6136 were found to be at reduced risk of developing cachexia as defined by weight loss >10% (main study (OR 0.52, 95% CI 0.29–0.93, $p = 0.026$); validation study (OR 0.09, 95% CI 0.01–0.98, $p = 0.035$)). We were unable to confirm other significant associations from the main cohort in the validation study. This may be due to the small sample size of the validation study which is a key limitation.

This study included a variety of cancer types, with significant numbers of patients with cancers of the digestive tract, lung and pancreas. Validation in larger independent cohorts is required to fully establish the generalizability of our findings, however the significant association with the rs6136 polymorphism and cachexia across both the main group and an independent validation cohort suggest that our results may apply across numerous cancer types.

Due to the small sample size of the validation cohort, we chose only to perform gene group analysis on the main cohort. The gene group analysis performed provides one way of summarizing the evidence between cachexia traits and multiple genetic variants across groups of genes that share functional similarity. Appetite regulation was found to be most significantly associated with the cachexia trait weight loss >15% and CRP >10 mg/l ($p=0.008$). There has been some evidence to date that negative regulators of appetite are elevated in cachexia (Doehner et al, 2001; le Roux et al, 2005). A number of animal studies have also shown prevention or reversal of cachexia by deletion or blockade of specific appetite pathways (Marks et al, 2001; Nicholson et al, 2006; Wisse et al, 2001).

In addition to the above link, the glucocorticoid signalling pathway was also found to be associated with cachexia (weight loss >15% and CRP >10 mg/l) ($p=0.0181$). There has been evidence that glucocorticoids and its associated signalling pathway are involved in accelerating protein degradation in muscle, which results in loss of lean body mass in cachexia (Tisdale, 2009). Glucocorticoids work through a permissive effect on the upregulation of messenger RNA and the subsequent synthesis of components of the ubiquitin–proteasome system in muscle. Glucocorticoids inhibit protein synthesis and promote gluconeogenesis, and suppress glucose and amino acid muscle uptake by inhibiting cellular transporters (Lecker et al, 2006). Mitogen activated protein kinases (MAPK) activity regulation was also found to be associated with cachexia (weight loss >15% and CRP >10 mg/l) ($p=0.0264$). MAPKs are known to mediate lipolysis in cancer cachexia (Ryden & Arner, 2007), and are also potential regulators of muscle catabolism in cachexia (Keren et al, 2006).

Previous genetic studies on cancer cachexia have identified associations with cachexia and polymorphisms in cytokine genes such as the *IL1B* 3954C/T polymorphism (rs1143634) in patients with gastric cancer (Zhang et al, 2007), and the *IL10*-1082A/G polymorphism (rs1800896) in patients with gastro-oesophageal cancer (Deans et al, 2009a). Cancer related anorexia has been associated with the *TNF*-308G/A polymorphism (rs1800629) in patients with non-small cell lung cancer (Jatoi et al, 2009). Despite some significant associations with other polymorphisms in pro-inflammatory cytokines genes (Table 2), we were unable to confirm the previous specific associations in the present study. However, all these studies have focused only on one particular type of cancer and on a small number of genetic variants. More widely applicable biomarkers may prove more useful. One of the strengths of the present study is the analysis of a wide variety of candidate genes that may influence the development of cachexia in patients with various cancer types.

The nature of cancer cachexia dictates that there are fewer individuals who develop the most severe aspects of the syndrome. At the severe end of the cachexia spectrum, the power in the present study to detect weak associations with uncommon variants was low. It may be that a larger sample size may be required to fully elucidate the effects of such variants in individuals with severe or refractory cachexia.

The diverse cachexia phenotypes we investigated represent various stages in the cachexia journey with potential genetic influences at each stage. The present study suggests that multiple pathways are likely to be involved in the pathogenesis of cancer cachexia and, in particular, P-selectin, appetite regulation, glucocorticoid signalling and MAPK activity regulation may have central roles in this process and should be further investigated. The animal data presented herein suggests that upregulation of P-selectin in skeletal muscle accompanies muscle atrophy in different circumstances. It remains to be determined if modulation of P-selectin might alter the development of cachexia in such models and therefore be a candidate therapeutic target in human cancer cachexia.

MATERIALS AND METHODS

Main study population

Study subjects were recruited from three centres from 2004 to 2008: NHS Lothian, UK; Cross Cancer Institute, Edmonton, Canada; and McGill University Health Centre, Montreal, Canada.

All subjects recruited had participated in clinical or research studies at the host institutions under ethically approved protocols. Recruitment was conducted at first presentation to surgical or oncology clinics at each institution. Recruitment was performed sequentially with the following exclusion criteria: (i) under 18 years of age; (ii) learning disability, and mental health problems; (iii) inability to give written, informed consent; (iv) presence of underlying infection; (v) on corticosteroids.

Patients recruited generally had cancer types with propensity to develop cachexia (*e.g.* gastric/oesophageal, pancreatic, lung). Overall, 855 patients were recruited. More than 98% of the study subjects were of European descent. Information collected on each patient included date of birth, date of diagnosis, type and stage of cancer. All patients underwent measurements of height and weight at the time of recruitment to the study. Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. Although there may be recall bias, evidence to support the reliability of self-reported weight and weight history (Perry et al, 1995; Stunkard & Albaum, 1981) is well documented. Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost. Height and weight data were subsequently used to compute a common anthropometric descriptor, BMI (kg/m^2).

Serum CRP concentration was measured with an automated immunoturbidimetric assay by each institution's clinical chemistry department using blood collected from patients at the time of recruitment and before any therapeutic intervention. CRP measurement was not available from patients recruited from the Cross Cancer Institute, Edmonton, Canada.

Stage of disease was based on the American Joint Committee on Cancer stage groupings I, II, III and IV.

All patients provided written informed consent to allow analysis of their DNA.

Phenotype definitions

There is currently no consensus diagnostic criteria for cancer cachexia, however two recent international consensus groups (Evans et al,

2008; Fearon et al, 2011) provide a conceptual framework for the classification of this condition. Cachexia is defined by the presence of involuntary weight loss. Varying thresholds of weight loss have been used, the most common being >5% (Fox et al, 2009; Knoll et al, 2008; Maltoni et al, 2001) and >10% (Gordon et al, 2005; Skipworth et al, in press; Zhang et al, 2007). A weight loss of >15% has been linked to major complications in cancer patients undergoing surgery (Antoun et al, 2009). Evans et al (2008) suggested classifying cachexia as mild or greater, moderate or greater or severe depending on whether the observed weight loss is >5, >10 or >15%, respectively.

The presence of underlying disease and pro-inflammatory catabolic signals discriminate cachexia from malnutrition (Evans et al, 2008; Fearon et al, 2011). The presence of systemic inflammation (serum CRP >10 mg/l) has also been linked to decreased survival (Mahmoud & Rivera, 2002; McMillan et al, 2003), and has also been correlated positively with weight loss in human cancer patients (Deans et al, 2009b; O'Gorman et al, 1999). CRP was incorporated into a three-factor model of cachexia for patients with pancreatic cancer (Fearon et al, 2006). The latter multi-profile definition was found to have more prognostic value compared with weight loss alone.

To take into account the above, we classified cachexia as a spectrum, represented by cut-offs of >5, >10 and >15% weight loss and we also examined weight loss in the presence of systemic inflammation.

Candidate gene and SNP selection

Initial candidate gene and SNP selection was based on a systematic literature review of SNPs with either putative functional or clinical relevance in the development of cancer cachexia (Tan et al, 2011). A further 18 candidate genes were selected based on the results of a gene expression analysis array study on muscle samples of cancer patients with cachexia (Stephens et al, 2010). From these genes were selected non-synonymous coding SNPs with MAF of >0.05. Overall 191 SNPs in 99 genes were considered for the association study.

Genotyping

The Applied Biosystems SNPlex™ Genotyping System (Applied Biosystems, California, USA) was employed for SNP genotyping. All DNA samples were processed and assayed without regard to phenotype. DNA samples were separated electrophoretically on a 3730 DNA Genetic Analyzer (Applied Biosystems, California, USA), and automated allele calls and genotype clustering of each individual sample was performed by Applied Biosystems' GeneMapper® Software (version 4.0). All automatic calls by the software were evaluated by one researcher. Any SNPs with less than 90% of the sample auto-called by the software were either rescored manually or discarded if clustering confidence was low. Reproducibility was determined by rerunning entire plates of DNA samples and a reproducibility rate of 99.7% was achieved.

Individual samples were removed if more than 10% of SNPs failed genotyping, and individual SNPs were removed if more than 10% of samples failed. As an additional genotyping quality-control check, SNPs with significant deviation from Hardy–Weinberg equilibrium (HWE) ($p < 0.01$) were removed from the final analysis. SNPs with a MAF <0.03 were also removed from the final analysis.

Power calculations

Power calculations were performed using Quanto. For the most prevalent cachexia phenotype (*i.e.* >5% weight loss, 54% affected), the present study has between 43 and 97% power to detect an OR of 1.5 for SNPs with a MAF of 0.05–0.35.

For the least prevalent cachexia phenotype (*i.e.* >15% weight loss & CRP >10 mg/l, 14% affected), the present study has between 12 and 40% power to detect an OR of 1.5 for SNPs with a MAF of 0.05–0.35.

Statistical analysis

Statistical analyses were performed using PLINK (version 1.06) (Purcell et al, 2007). Patients who met the criteria for each of the proposed cachexia phenotypes were compared with patients who have lost ≤5% body weight as control. Unconditional logistic regression was employed to calculate ORs and their 95% CI for the minor allele of individual SNPs and its association with each proposed cachexia phenotype. All analyses were adjusted for covariates that may affect weight loss, *i.e.* age at diagnosis, sex, pre-diagnosis BMI, tumour type and stage.

To account for multiple testing, permutation testing was performed by running the adaptive permutation test in PLINK within each proposed phenotype. Permutation tests are often employed to adjust groups of correlated tests for multiple testing, since conventional methods such as Bonferroni correction are overly conservative when tests are correlated (Conneely & Boehnke, 2007). The adaptive permutation test in PLINK gives up permuting SNPs that are clearly going to be non-significant. This greatly speeds up the permutation procedure, as SNPs that are not significant will drop out quite quickly, making it possible to properly evaluate significance for the handful of SNPs that require millions of permutations.

SNPs with a permuted p-value of <0.02 within the same chromosomal region (within 10 000 kb) were then analyzed for any possible haplotype associations. Only haplotypes that had a frequency greater than 5% were considered for further analysis. Each identified haplotype and significant SNPs were then tested for association with percentage weight loss as a continuous variable.

Finally, candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology using AmiGO (Supporting Information Table S2). The set-based test in PLINK was used to analyze association between grouped SNPs and cachexia phenotypes. The set-based test selects the best set of SNPs whose mean of these single SNP statistics is significant after permutation, which is particularly suited to large-scale candidate gene studies (Ott & Hoh, 2003). The empirical p-values of the set-based test were obtained by a permutation of 10 000 times of phenotype labels.

Validation study

Subjects from the validation study were recruited from an independent centre, Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland from 2007 to 2008. All patients with proven cancer diagnosis were considered. Patients were recruited sequentially at first presentation to the oncology clinic. Exclusion criteria were identical to the main study.

In total, 101 cancer patients were recruited, all of whom were of European descent. Like the main study, all patients underwent measurements of height and weight at the time of recruitment. Premorbid weight was recalled by the patient and verified where possible from the medical notes. Individual weight loss was calculated and expressed as percentage of pre-illness body weight lost. Height and

The paper explained

PROBLEM:

More than half of cancer patients suffer from cachexia, and it is responsible for death in up to 20% of cases. Cachexia is also a significant cause of morbidity in cancer patients. Based on our current knowledge of demographic and clinical factors, we are unable to predict, for any given cohort of patients, who will develop cancer cachexia and who will not. Such variation may, in part, be due to the patient's genotype. Knowledge of genotypic variation associated with cachexia would contribute to early identification of patients at risk and allow institution of prophylactic measures.

RESULTS:

In a large scale genetic association study, the C allele of the rs6136 (P-selectin) SNP was found to be associated with weight loss >10% both in the discovery study and the validation study. To further corroborate the P-selectin SNP to cancer cachexia in the gene association study, we tested the same gene for participation in the induction of the skeletal muscle atrophy gene program in animal models of cachexia. P-selectin was found to be significantly upregulated in muscle following both tumour-induced cachexia in rats and intra-peritoneal injection of LPS in mice.

IMPACT:

The C-allele of the rs6136 polymorphism is associated with reduced risk of developing cachexia. Identification of P-selectin as relevant in both animal models and in cachectic cancer patients supports this as a risk factor/potential mediator in cachexia.

weight data were subsequently used to compute a common anthropometric descriptor, BMI (kg/m^2). Serum CRP concentration was measured with an automated immunoturbidimetric assay at the institution's clinical chemistry department using blood collected from patients at the time of recruitment and before any therapeutic intervention.

Patients were genotyped for SNPs found to have permuted $p < 0.05$ in the main study and quality control checks were carried out as described previously. As with the main study, patients in each of the proposed cachexia phenotypes were compared with patients with ≤5% weight loss as control, and association analyses were adjusted for age at diagnosis, sex, pre-diagnosis BMI, tumour type and stage.

Animal studies

Wild type C57BL/6J mice (20–25 g) (Jackson Laboratories) and male F344/NTacfBR rats were maintained on a normal 12:12 h light/dark cycle and provided *ad libitum* access to water and food. Animals were anaesthetized at the time of tumour implantation or sacrifice using a ketamine cocktail. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committees of Oregon Health & Science University.

Intra-peritoneal injection of LPS

Lipopolysaccharide was dissolved in 0.5% bovine serum albumin (BSA)/0.9% saline and injected intra-peritoneally at 250 μg/kg. Food was removed from cages at the time of injection, and animals were sacrificed 8 h after injection.

Cancer cachexia model

The MCA sarcoma does not metastasize and has a curvilinear growth pattern (Sato et al, 2001). On day 0, TB rats ($n = 8$) had 0.2–0.3 g tumour tissue implanted subcutaneously into the flank (Ramos et al, 2004) whilst controls ($n = 7$) underwent sham operation (SH). On day 13, tumour growth was within the pre-determined end-points of the study, according to OHSU IACUC Policy on tumour burden and the animals were sacrificed. Body composition was determined by magnetic resonance (EchoMRI, Echo Medical Systems, Houston, TX) at the time of tumour implantation and again at the time of sacrifice. The gastrocnemius muscles were immediately removed, weighed, preserved in RNAlater solution (Ambion, Inc.) and stored at −80°C until RNA extraction and qPCR analysis.

qPCR analysis

Total gastrocnemius muscle RNA was extracted using the RNeasy fibrous tissue mini kit (Qiagen, Valencia, CA). The total RNA was quantified and checked for integrity using standard protocols. Complementary DNA (cDNA) was transcribed using Taqman reverse transcription reagents according to the manufacturer's instructions. PCR reactions were run on an ABI 7300, using Taqman universal PCR master mix, using Taqman gene expression assays. Relative expression was calculated by the ΔΔCt method using GAPDH as an endogenous control.

Author contributions

KCHF, FSk, VEB, DLM, SK and JAR designed the study. BHLT, AV, FSt, DACD, RJES, SD and TSS recruited the patients and collected data. BHLT and TF performed the genotyping and overall genetic analysis. TPB performed the animal studies. All authors contributed towards the interpretation of the data, critically reviewed and commented on the report and approved the final version.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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